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EDITOR'S VIEWPOINT

Crazing of acrylic teeth and dentures is observed more commonly when dentures are processed against tin-foil substitutes than when tin foil is used. Sweeney, Brauer, and Schoonover (p. 306) found that increased water sorption at curing temperatures is an important factor in crazing. An explanation of crazing and of blushing is proposed. Cross-linked polymethyl methacrylates, commercially available, are craze-resistant.

Crawford and Larson (p. 313) have developed a method for determination of residual mercury in dental amalgams. It is more rapid and accurate than other methods and should provide a means of investigation for the smaller laboratory, as well as for those more fortunately equipped.

Shiere and Manly (p. 318), from a study on 250 adolescents, found that at 17 years of age masticatory efficiency is comparable to that of young adults. The loss of one molar resulted in reduction in efficiency to one-half. This is highly significant when the early loss of first molars is considered. Since efficiency is drastically reduced at an early age, there may be greatly increased stress on remaining teeth to compensate for this defect.

Clark, Tam, and Mitchell report "takes" in all of 19 instances of transplantation of developing teeth from the third molar to the first molar region of 13- to 23-year-old patients. Teeth also have been transplanted to extraoral sites in animals (see following) and to tissue culture with success by others. In this study, it is most interesting that the pulps gave positive reactions to pulp testing in 43 per cent by the fifth month and in 85 per cent by the third month. While the practicability of this technique remains to be established, this is a most encouraging report.

Fleming (p. 329) successfully transplanted human tooth germs to the anterior chamber of the eye or the axilla of guinea pigs, dba mice, and rabbits. While the host originally serves as a "tissue culture medium," the transplant soon becomes vascularized and is then considered part of the host. The brain was not receptive under the conditions of the study. It now appears well established that the tooth germ will survive transplantation and it is hoped that such transplants may afford information on growth and development not readily observable in other material.

Grand (p. 341) used tissue culture growths of heart and kidney tissue from chick embryo to test the relative effects of sodium fluoride and sodium oxalate. Sodium fluoride was two and one-half times more toxic than sodium oxalate. The immobilization of calcium did not appear to be the critical factor. The inhibition of enzyme systems is not overlooked but is not discussed. The interpretation of this study requires more understanding of the action of ions, such as oxalate and fluoride ions, on cells and tissues.

Hatton, Nebergall, and Muhler (p. 350), from further studies using stannous fluoride and sodium fluoride solutions with powdered enamel, show that more stannous fluoride is taken up than sodium fluoride. They suggest that the pH of the solutions may be a factor.

McClure and Folk (p. 358), feeding experimental cariogenic diets to rats, observed important differences referable to the methods of feeding and housing. The size of the group in each case, the freshness (or fecal contamination) of the diet and, as previously demonstrated, the age of the animals at initiation of the diet, all influence the caries rates. Evidence continues to demonstrate that studies of caries in experimental animals is neither easy nor simple. The animals' genetics, age, housing and feeding conditions, and small differences in food (see following) and water composition are among the many factors to be considered. The present study was designed to further test the cariogenicity of certain processed foods.

The investigation of dental caries in the rat is complicated by many small facets. Muhler (p. 364), investigating the effect of different experimental diets, found 46 per cent corn grits and 19.5 per cent sucrose very effective for inducing and extending carious lesions in the rat. The use of coarsely ground corn in place of corn grits and sucrose reduced the average caries severity by practically 50 per cent. It is interesting that different constituents produced different

caries' pictures as well as different types of caries. This sensitivity of the rat to variations in experimental diets is a problem that must be considered by any investigator who is studying the effect of diet and nutrition on dental caries in this animal.

Snyder, Hackedorn, Martin, and Johnson (p. 368) found two bacteria which commonly inhabit the mouth to be capable of producing mucinous polysaccharides from sucrose. *Streptococcus salivarius* produced levan, and gram-negative diplococci produced amylopectin. Large numbers of *Str. salivarius* were found in saliva regardless of caries activity. Although the authors undertook this investigation in relation to dentobacterial (mucinous) plaques, the water-soluble nature of the mucopolysaccharides was believed to limit their possible role in plaque formation.

Clapper and Heatherman (p. 380) investigated the metabolic activity of two strains of lactobacilli which appeared in different quantities in mouths of individuals with extensive or little caries. Rhamnose-fermenters (Type I), previously found with more extensive caries, produced more acid and grew faster than rhamnose-nonfermenters (Type II). Other tests, including production of reducing sugars from sucrose, help to establish these as distinct types. Placing NaF in the media reduced the acid production, growth rate, and glycolytic activity of Type I. The differentiation of types of lactobacilli and influences on their metabolic activity is of importance to investigators of the caries process and of anticaries measures. The definite effect of NaF on bacteria, again shown here, may represent a useful approach to the modus operandi of the caries-fluoride effect.

As the inadequacy of the cavity count as an indicator of caries activity becomes more generally recognized, the need for a simple test for caries susceptibility becomes more evident. Muracciole (p. 387) suggests a rather simple colorimetric test of salivary buffer capacity but, unfortunately, has evaluated it against DMF. It should be evaluated against new cavity formation.

Hurtarte and Scrimshaw (p. 390) found a low caries activity as indicated by DMF studies among children of Guatemalan villages high in the mountains. Dietary studies showed a deficiency of vitamin A, carotene, and animal proteins. Their food is largely tortillas prepared from corn soaked in lime. They have little refined carbohydrate. Observations indicate that the adults have poor dental conditions, which are attributed to accumulation of untreated dental lesions.

Hatton's (p. 397) study of the eruption data of anterior teeth of monozygotic and dizygotic twins permitted the estimate that genetic factors have a 78 per cent influence on eruption time and environmental factors only a 22 per cent influence.

Berliner (p. 402) has evaluated three aqueous iodine solutions on 168 patients with marginal gingivitis, 43 with necrotizing ulcerative gingivitis, 192 with periodontitis, 21 with aphthae, and 9 with traumatic ulcers. The use of a "rubber sleeve" isolating the teeth of one jaw aided in evaluating results of those agents in the same patient. Berliner's results indicate that a solution of diatomic iodine is more effective than other iodine solutions to aid in treatment of marginal gingivitis, necrotizing ulcerative gingivitis, aphthae and traumatic ulcers, and to some extent in treatment of periodontitis. A therapeutic agent may aid by antibacterial action and by promoting healing but is not a substitute for removal of irritants and tissue reactions.

Olsen, Teuscher, and Vehe (p. 413) found the innervation of the upper anterior teeth to be quite variable. The origin of the anterior superior alveolar nerve is dependent upon the number of branches (1 to 3) arising from the infraorbital nerve to form it. The number of nerve filaments arising from the anterior superior alveolar nerve is far from constant. In about one-half of the 26 maxillae studied, there was no middle superior alveolar nerve. The nasoalveolar nerve did not enter the pulp of the first incisor in any of the specimens. It is suggested that anesthesia of the anterior maxillary teeth by means of the incisive canal injection is obtained because of diffusion of the anesthetic agent through the cancellous bone.

Chilton (p. 421) describes the use of the Latin square design for clinical experiments. He uses, as an example, the problem of evaluating drugs proposed as relaxants during operative procedures for cerebral palsy children. The method can be applied for evaluations of various drugs in relationship to clinical problems. The paper is presented in a manner that makes it useful to the investigator not trained in statistical methods.

The need for a relatively inexpensive biologic tool for studying the effects of fill-

ing materials and other agents used in cavity preparation and restoration prompted Maurice and Schour to develop a method using the rat molar (p. 429). This technique may afford a relatively simple assay of filling materials and other agents but whether the results are comparable to effects in human beings should be established.

Fritz (p. 435) investigated protein metabolism of salivary glands using isotope-labeled glycerin on rats. The concentration of salivary gland proteins was below but paralleled liver protein levels. Differences were observed between parotid and submaxillary protein concentrations. This approach to the problem of salivary gland metabolism has not yet advanced to the stage where interpretations are justified, but it offers the possibility of yielding important information.

Rats fed labeled glycerin were investigated for protein uptake and turnover in the gingival mucosa, palatal mucosa, tongue tissues, and incisor pulp. Fritz (p. 445) found the turnover rates to be more rapid than for those of the liver in all except pulp tissue which equaled that of the liver. He interpreted the rapid initial uptake of glycerin by the palatal mucosa as correlated with its rapid healing rate. Unfortunately, from this concept, the other tissues in decreasing order of initial uptake are tongue membranes, liver, pulp, gingival mucosa, and muscles. These probably do not represent the order of healing ability.

Auskaps and Shaw (p. 451) injected trypan blue into rats and monkeys and investigated its disappearance from the blood serum and the vital staining of bones and teeth. As would be expected from previous studies, the forming bone and dentin are deeply stained with trypan blue and the nonmesenchymal enamel is not. The trypan blue appears bound to organic factors as opposed to alizarin red S which is bound to fractions removed by decalcification. A combination of the two techniques of vital staining should be useful in studies of odontogenesis.

Waerhaug (p. 458) has used a technique of boiling specimens in celloidin to hasten embedding. Anyone who has worked with the celloidin technique, or has waited for specimens being processed, will welcome this timesaving method.

Hess and White (p. 462) developed a somewhat simplified method for determination of citrate in small quantities of bone and tooth tissue. It is also applicable to other materials.

In this issue of the JOURNAL the first fruits of the campaign for supporting associates are seen as extra pages. The contributions of these associates will permit the publication of at least 64 additional pages this year and will pay the expenses of the offices of the Editor and the Business Manager. When the present contract for the JOURNAL was consummated, the publishers agreed to pay a sum annually to the Association, which was to be used for expenses, and agreed to publish 900 pages per volume.

It has become apparent that the number of subscribers to the JOURNAL will remain relatively small and that advertising will not be very profitable. In view of this, the publishers were released from their obligation to pay a sum, annually, to the Association. Coupled with the small subscription list is a paradoxical increase in dental research activity and therefore in contributed articles. Comparison of the "date received for publication" on each paper and of the actual publication date will indicate the delay caused by a large backlog of papers. This is the result of increase in receipt of acceptable papers and maintenance of the status quo in the number of available pages.

It has been proposed that the editorial policy be made more stringent and thereby eliminate many papers. We decline to judge the *future* value of *present* research and prefer to follow the original policy of publishing material if it appears to be the result of honestly conducted research with properly documented conclusions. We hope that our sustaining associates will continue to aid us by affording more space for publication of such material. Their contribution to research is motivated by desire to support an important facet of dental research, publication.

The American Association for the Advancement of Science will sponsor the 1955 Gordon Research Conference on the Structure, Chemistry and Physiology of Bones and Teeth, August 1 through August 5. Dr. W. F. Neuman will serve as Chairman and Dr. R. F. Sognnaes as Vice Chairman. Interested individuals may arrange to participate and receive additional information from Dr. George W. Parks, Colby Junior College, New London, New Hampshire.

H. B. G. R.

CRAZING OF ACRYLIC RESINS

W. T. SWEENEY,* G. M. BRAUER,** AND I. C. SCHOONOVER***

National Bureau of Standards, Washington, D. C.

INTRODUCTION

APPEARANCE of small surface cracks is often observed in dentures and plastic teeth. These cracks lower the esthetic qualities of the restorations and greatly reduce their strength. Crazing is the result of local stresses set up in the resin. When the stress exceeds that necessary to fracture the resin, crazing occurs. The object of this investigation was to determine the factors which produce crazing in dental acrylic resin and to explain the crazing mechanism.

Typical photographs of crazing in a denture and in plastic teeth are shown in Figs. 1 and 2. It has been shown[1-4] that crazing in polymethyl methacrylate is caused either by application of external mechanical stresses or by the action of chemical agents on stressed material (solvent crazing). The latter type of crazing may occur in the presence of residual monomer, solvents, or depolymerization products. These substances will produce local strains greater than the material can withstand. Often the simultaneous action of solvents and external stresses produces crazing. It is probable that the crazing of dentures results mainly from solvent action in combination with both internal and external stresses.

Examination of dentures in service and of laboratory specimens showed that there was a wide variation in the amount of crazing occurring in denture bases and in plastic teeth made of the same brands of material. It was also observed that plastic teeth which did not craze on the application of a solvent before being placed on a denture were often susceptible to crazing after being subjected to the denture processing procedure. Because these observations indicated that the craze susceptibility of the resins was dependent upon variations in the processing procedure, the effects of several of these variations were investigated. Included were the effect of the type of investment or mold material used in processing the denture, the effect of water sorption during processing, and the effect of the molecular weight of the resin.

CRAZING TESTS

The "standard" craze test selected for determining the craze susceptibility of the resins consisted of 3 cycles of alternately immersing the specimen

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in methyl methacrylate monomer for 5 seconds and then drying it in air for about 30 seconds. Specimens which were crazed by this standard procedure also crazed on the application of ethyl alcohol and many other solvents.

To determine the effect on crazing of stresses produced because of the differential thermal expansion between resin teeth and investing media, teeth were invested in a number of materials varying widely in thermal expansion and in strength. Teeth invested in dental stone, dental investment containing silica gel, Boilsoft investment, and alginate impression material were held in a water bath at 100° C. for one-half hour. After cooling, the teeth were subjected to the standard craze test and crazing was observed in all cases regardless of the type of investing material used. The uniformity of crazing observed

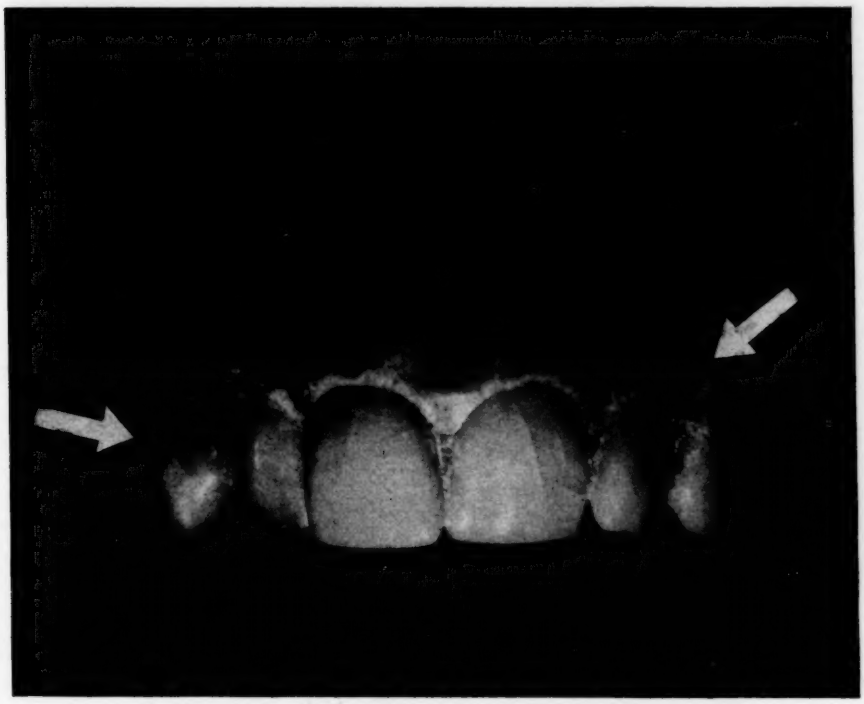

Fig. 1.—Typical crazing of acrylic denture.

after investment of the resins in materials varying so widely in physical properties strongly suggested that differences between the thermal expansion of the investing media and the resin were not major factors in crazing.

The results of experiments in which teeth were maintained at 100° C. by technics which prevented their coming in contact with water indicated that water sorption was a major factor in the crazing of teeth. Teeth heated in air gave no craze pattern. Teeth invested in Acrawax C (melting point 137° C.) and then suspended in a water bath at 100° C. for 3 hours showed no craze marks on application of the craze test. Teeth heated in stone investment from

which free water had been removed by drying in vacuum and heating to 110° C. did not craze. Acrylic dentures polymerized at 71.1° C. and 100° C. against tin foil–lined plaster molds did not craze, whereas those prepared against alginate tin foil substitutes invariably blushed and showed extensive craze patterns. It has been reported[5, 6] that water from the plaster or stone penetrates the resin when it is polymerized against alginate-separating media, which are water permeable. Sweeney[5] showed that a loss in weight on drying

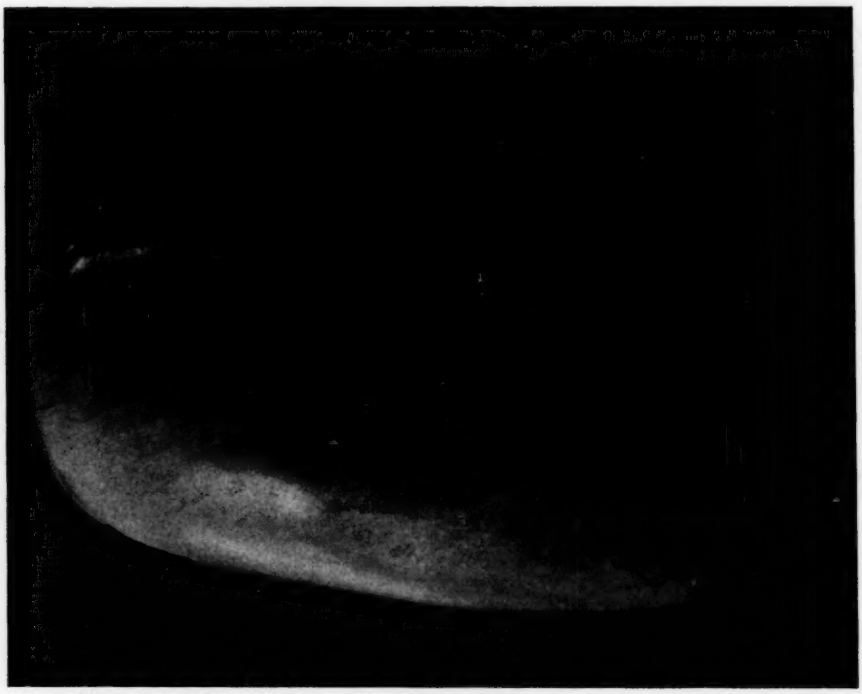

Fig. 2.—Crazing of acrylic tooth.

of resin disks cured against alginate base material was 2.7 per cent whereas tin foil–lined specimens lost only 0.6 per cent. This loss of water was accompanied by volume shrinkage. The effect of water sorption during processing was illustrated with sheets of resin 1.5 mm. thick polymerized against alginate on one side and against tin foil on the other. When these specimens were dried, the side polymerized against the alginate shrank more than the side cured against tin foil because of the greater loss of water, and the specimens warped.

Crazing was found to be more pronounced when denture specimens were allowed to dry for a few hours after processing before being subjected to the crazing test. Furthermore, if an increased amount of water was introduced into the specimen during processing (for example, by processing the resin in an autoclave), more severe crazing resulted.

In order to determine the effect of water temperature on the water sorption and crazing, measurements of the water sorption of 2 polymethyl methacrylate disks (0.4 mm. thick), which were molded in a Carver press at 140° ± 5° C. and 10,000 p.s.i. from duPont HG_4 (fine) molding powder, were made at various temperatures. Samples were stored in water and weighed at various time intervals. Specimens kept below the second-order transition temperature, T_g (that is, the temperature in the 55° C. to 80° C. range at which a relatively abrupt change takes place in the mechanical, thermal, electrical, and optical properties of the polymer), reached approximate sorption equilibrium (attained constant weight) within less than ten days. Water sorption below T_g was nearly constant but increased markedly above the second-order transition point (Fig. 3). In this figure water sorption at 100° C. does not represent the equilibrium value. The disks still increased in weight after 200 days of water exposure.

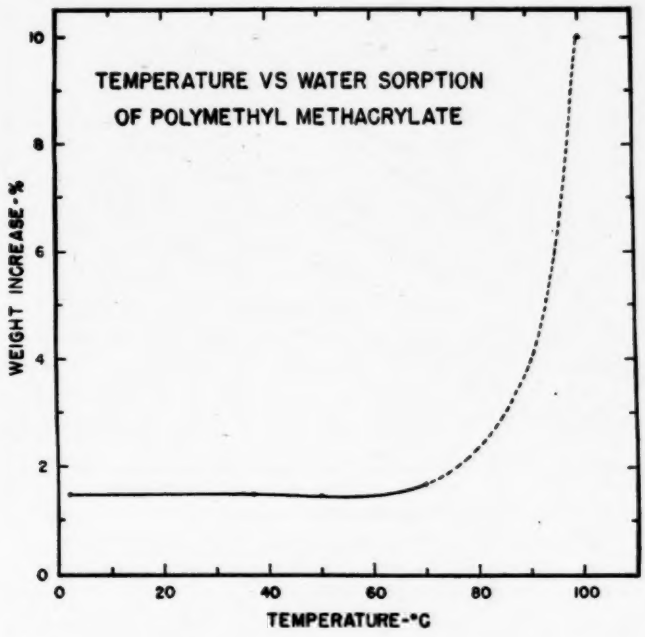

Fig. 3.

Several Plexiglas II-UVA sheets 1.8 mm. thick were immersed in water for a few days and subjected to the craze test. Crazing occurred (using the standard test at room temperature) for samples which had been immersed in water at 100°, 70°, 50°, and 37° C. Thus, crazing occurred for samples which had been immersed in water above as well as below T_g. Specimens stored at 4° C. did not craze. As can be observed from Fig. 3. which shows the water

sorption–temperature relationship, lack of crazing at 4° C. cannot be attributed to difference in water sorption. Increase in craze resistance with decrease in temperature has also been observed by Maxwell and Rahm for polystyrene.[7]

EFFECT OF MOLECULAR WEIGHT ON WATER SORPTION AND CRAZING

On storage of specially prepared polymethyl methacrylates* of various molecular weights in water at 100° C., which is well above T_g, a continuous weight increase was observed even after 100 days (Fig. 4). The rate of water sorption and increase in weight decreased with increasing molecular weight. Depending on the viscosity molecular weight, water sorption values of the order of 2 to 10 times those found at room temperature were obtained.

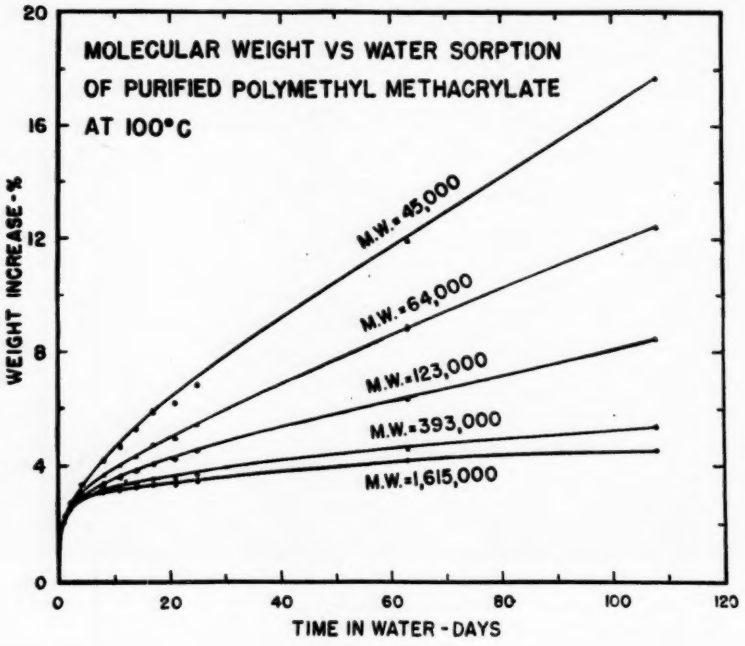

Fig. 4.

It was observed that crazing is affected by the molecular weight of the specimen (Fig. 4). Three samples of each of 5 different viscosity molecular weight materials (45,000 to 1,615,000) were given the test for crazing after being heated for 1 hour in water. The higher molecular weight material was more craze resistant than specimens of lower molecular weight. This may be due to the smaller water sorption of the higher molecular weight specimens as well as the higher strength of the higher molecular weight material.

The number average molecular weight for tin foil–cured samples as measured by the method of Caul and Schoonover[8] ranged from 27,000 to

*The authors wish to thank W. F. Bartoe of the Rohm and Haas Co. for supplying the samples.

29,000, whereas material cured against tin foil substitutes which are water permeable had molecular weights varying from 36,000 to 38,000. Due to the spontaneity of the polymerization reaction, the molecular weight variations are not considered significant. These results show that crazing of dentures packed with tin foil substitutes is not due to a lower molecular weight of the denture resin.

BLUSHING

Blushing (evidenced by a hazy appearance due to light scattering) occurred when a Plexiglas sheet which had been dipped into monomer was exposed to water-saturated air. The bleached or milky white portion extended to a very shallow depth as evidenced by the disappearance of blushing on removal of a thin surface layer. Examination under the microscope showed no distinct cracks at a magnification of 500.

When a specially prepared sample which showed both blushing and crazing was heated slowly to 150° C., blushing and craze marks disappeared and did not return on cooling. Specimens annealed in this manner showed no crazing when subjected to the standard crazing test.

MECHANISM OF CRAZING

It is evident that a complete description of the mechanism of crazing is not possible from data available at this time. The observed effects, in our opinion, may be explained as follows:

During processing the denture base material is heated above the transition temperature. After the polymerization is complete and while the polymer is still above T_g the chains of the polymer are less rigidly interconnected. The chain network is expanded as compared to its normal arrangement below the transition temperature. This allows the water molecules to permeate more easily between the polymer chains, and an increased quantity of water is absorbed. Greater water sorption above T_g may also be caused by a possible increase in hydrogen bonding due to the presence of small amounts of depolymerization and hydrolysis products at elevated temperatures, especially if the material is a copolymer of polyacrylic acid ester. On cooling below T_g, the resin becomes supersaturated with water. On placing the specimen in air, water and residual monomer evaporate from the surface. The shrinkage resulting from the evaporation of water and monomer from the outer layer or skin causes the surface layer to be in tension. When the strain is sufficiently large, fracture of the weak secondary bonds between the chains occurs and crazing cracks appear at right angles to the direction of stress. Often delayed fracture occurs because of the increase in magnitude of stresses produced by the continued evaporation of water. Craze marks appear after storing the resin for a few days. On subjecting the sample to the rapid craze test, crazing probably occurs by a mechanism similar to that described previously.

Whether the blushing of the material which takes place during the cooling period is due to a distinct precipitate phase or is caused by numerous submicroscopic openings which cause a large portion of the light striking them to

be reflected cannot be decided from the present investigation. Possibly, clues regarding this mechanism can be obtained from x-ray diffraction measurements and observations under the electron microscope. Samples of polymethyl methacrylate can be annealed in air at elevated temperatures. On heating above T_g, more and more segments of the molecule have sufficient energy to execute vibrations and rotations. Strains are released and blushing and craze marks are no longer visible to the naked eye. Furthermore, any water present in the polymer can diffuse more readily to the surface, where it evaporates.

Craze-resistant acrylic teeth are now commercially available. These teeth contain cross-linked polymethyl methacrylate of high molecular weight. The material is insoluble in most solvents, does not swell readily, and has a softening point above 130° C. In the preparation of the denture the teeth are probably not heated above the transition temperature. Also, reinforcement of the chains through cross-linking probably increases the amount of stress necessary to break chains. Hence, the craze resistance is increased through cross-linking.

SUMMARY

Investigations of the crazing of acrylic dentures and plastic teeth demonstrated that when linear methyl methacrylate polymer comes in contact with water during processing at elevated temperatures, the material is susceptible to crazing. Material protected from water during the processing does not craze.

An explanation for the crazing process, based on the experimental evidence, is advanced. Sorption of excess water at elevated temperature causes the surface of the material to be supersaturated with water when the specimen is cooled. Evaporation of excess water sets up strains which eventually are released by the formation of craze marks.

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RESIDUAL MERCURY DETERMINATION PROCESS

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IT IS generally agreed by the members of the dental profession that excess mercury remaining in a dental amalgam has a deleterious effect upon its physical properties. Compressive strength, flow, and setting changes are all adversely affected. The desirability of a quick, accurate method for mercury determination is quite apparent. However, practically all mercury analyses of dental amalgams have previously been made by a long and tedious wet analysis.[1] In the cases where the mercury was removed by heat from the amalgam and then collected to be analyzed, it seems most probable that there would be some loss of mercury before the wet analysis could begin.

Spectrographic analysis was considered briefly, but was ruled out as impractical. A trial plan was devised to heat amalgams of known composition to various temperatures and, by analytic weighings, to determine the degree of effectiveness the temperature was producing in ridding the sample of its mercury content. Various temperature and time combinations were used. Arbitrarily, 700° F. was selected for a trial run since that temperature was just above the boiling point of mercury.

Heating the amalgam to 700° F. for 15 minutes removed only 16.06 per cent of its mercury content. This was obviously not a fortuitous combination of time and temperature. Continued heating at this temperature for 1 hour released only 1 per cent more of the mercury, and after another 1½ hours of heating at this temperature only 1 per cent more mercury was removed. Continued work indicated that 1,000° F. for 1 hour was the most effective procedure. However, it became apparent that all the mercury was not being released or the alloy was increasing in weight by the formation of oxides which prevented gaining the desired accuracy and precision. To determine which was the cause, analytically weighed samples of mercury were placed in crucibles and heated to 1,000° F. for 1 hour. In these trials the method was 100 per cent effective in removing the mercury because coincident results were obtained by weighings prior to and at the conclusion of experiment.

Weighed samples of the alloys were subjected to the same treatment, and it was found that, as had been suspected, they were increasing in weight when unprotected at the elevated temperature. Therefore, it was concluded that an inert atmosphere would solve the problem.

EQUIPMENT

A heavy-walled Pyrex glass tube 4 feet long and 1¾ inch outside diameter is placed in a horizontal muffle furnace. One end of the glass tube has a one-hole rubber stopper connected by tubing to 2 Erlenmeyer flasks. The first of

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the flasks is empty and the second is filled with cold water. The second flask is connected to the vacuum outlet. The other end of the large glass tube is connected by rubber stopper and tubing to a tank of nitrogen (Fig. 1).

A stainless steel strip of metal 12 inches long and 1 inch wide and a supply of No. 000 Coors high form crucibles are also necessary for the test.

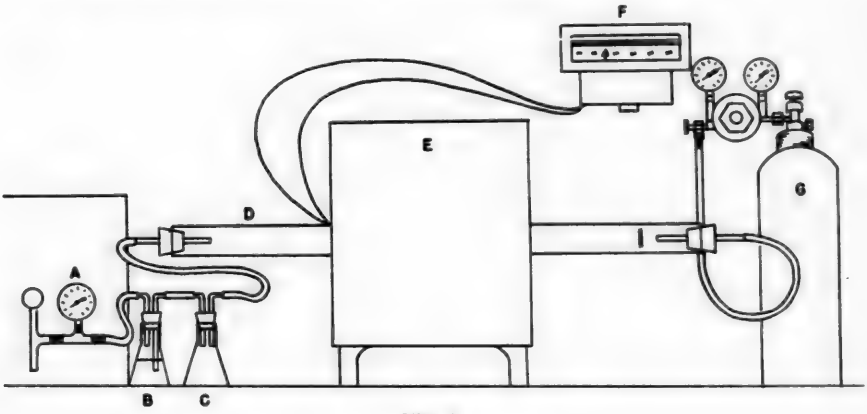

Fig. 1.

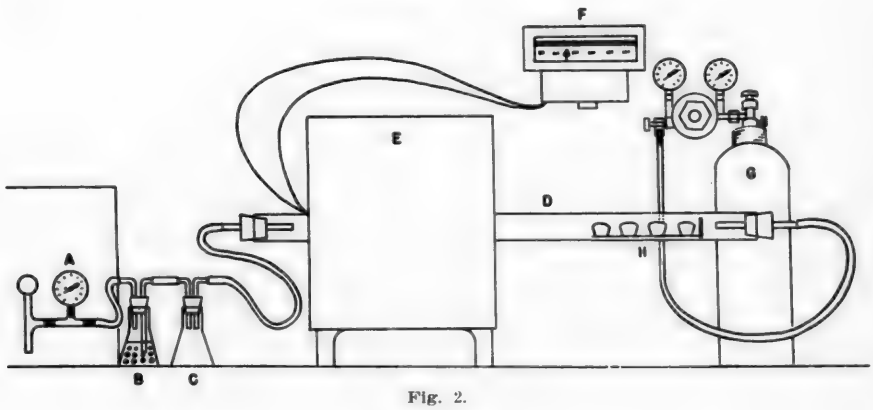

Fig. 2.

PROCEDURE

To run an analysis the amalgam sample is crushed into very small pieces in a swedger. The crushed amalgam is then placed in No. 000 Coors crucibles whose weights have previously been recorded. The crucibles with the samples are weighed on an analytic balance. Four crucibles containing samples are then placed on a stainless steel strip of metal and inserted in the large glass tube (1¾ inch diameter) which has been pulled out far enough on one side so that the metal strip is entirely out of the furnace (Fig. 2).

The ends of the glass tube are then closed with stoppers, and the vacuum is turned on so that all the air in the system is evacuated. Nitrogen is run into

the other end of the tube until the vacuum is filled. This evacuating and filling with nitrogen is repeated 3 times to ensure the absence of air from the system. The supply of nitrogen is diminished until only a few small bubbles of the gas are coming through the tube. The oven is then heated to 1,000° F. When this temperature has been reached the large glass tube is shifted so that the part of the tube containing the samples is in the constant temperature zone of the furnace (Fig. 3).

It is left in this position for 1 hour and the temperature is maintained. At the end of that time the furnace is disconnected and the glass tube is again shifted so that the portion containing the samples is free from the oven. The samples are allowed to cool in this position until they reach room temperature; nitrogen is still present. When the glass tube has reached room temperature the samples are removed and weighed immediately on the analytic balance.

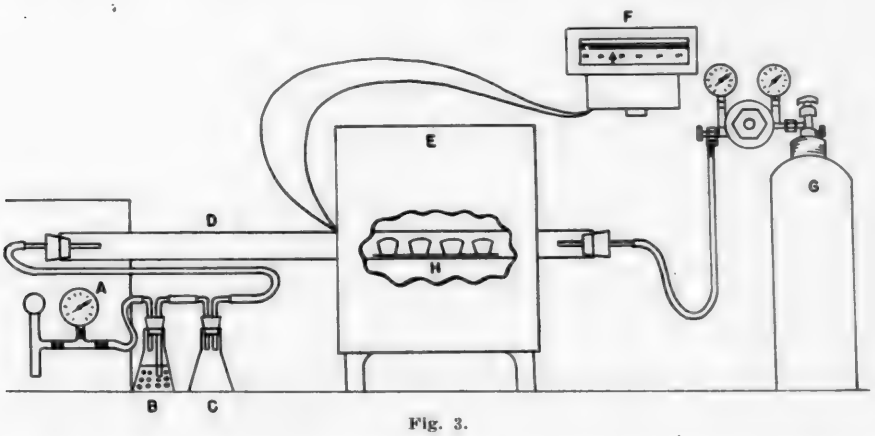

Fig. 3.

The amount of weight the samples have lost gives the mercury content of the amalgam sample from which the percentage of mercury that was present in the amalgam can be computed.

$$\frac{W_L}{W_O} \times 100 = \% \text{ Hg}$$

Where W_L is the weight loss of the amalgam sample and W_O is the original weight of the amalgam sample.

DISCUSSION

In the procedure described the glass tube is completely evacuated and filled with nitrogen to prevent formation of oxides, which would increase the weight of the sample and make an accurate determination of the mercury content impossible. The mercury that is removed from the amalgam condenses at the end of the glass tube, along the tubing, or in the first Erlenmeyer flask. Any mercury that is still in the vapor form is condensed on contact with the cold water in

the last Erlenmeyer flask. The empty flask (C) is a safety measure to prevent the water in flask (B) from being drawn into the hot glass tube if the nitrogen supply is stopped and a vacuum is again formed. The water into which the last of the mercury vapor condenses is a precaution against the dangerous fumes escaping into the room.

The optimum temperature of 1,000° F. and time of 1 hour were determined after many trials at various temperatures for different lengths of time. The temperature necessarily was kept well below the boiling points of the alloy constituents although above the boiling point of mercury[2] (Table I).

TABLE I

BOILING POINTS (° F.) OF ELEMENTS IN AMALGAMS

	Hg	Zn	Ag	Sn	Cu
Boiling point in degrees Fahrenheit	674.6	1,664.6	3,542	4,118	4,190

A lower temperature could be used but it would take considerably longer than the convenient 1-hour period. The Pyrex tube and the crucibles are not affected by the 1,000° F. heat but would not withstand higher temperatures.[3] This rules out the possibility of shortening the time by an increase in temperature.

It was found necessary to keep the samples in the tube while they cooled and to maintain the nitrogen supply so that the samples would not take on weight from the formation of oxides. This reaction can take place throughout the cooling period. If it is necessary to store the samples after they have been in the oven, but before they are weighed, they may be kept in a desiccator without changing their properties.

In order to check this procedure analytically weighed samples of mercury and weighed amounts of alloy were mixed in the mechanical amalgamator only long enough for all the mercury and alloy to combine without adhering to the sides of the container. Samples were then allowed to harden overnight before being crushed in the swedger. The crushed samples of amalgam were placed in the preweighed No. 000 Coors crucibles and weighed on the analytic balance. Since a known weight of mercury and alloy were used to form the samples, the percentage of mercury in the original sample was known and could be used to determine the gram weight of mercury present in the crushed sample to be tested. If θ is per cent mercury in original sample, and if W_t is weight of crushed sample to be tested, and if α is weight of mercury in sample to be tested, then,

$$(\theta)\ (W_t) = \alpha$$

The crucibles containing the samples were then placed in the Pyrex glass tube and the procedure described for testing was followed. When the cooled samples were weighed again on the analytic balance, the loss in weight of each sample was compared with the computed weight of mercury in that sample.

RESULTS

The results of our testing showed the accuracy of this procedure to be 0.039 per cent ± 0.016 average deviation (determined by standard mathematical procedure) when testing a variety of alloys—coarse, medium, and fine cut, rapid and slow setting.

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MASTICATORY FUNCTION OF ADOLESCENTS

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THE chewing ability of children 6 to 15 years of age has been reported.[1] On the average, the efficiency of the 6-year-old children was 40 per cent of an adult group taken as the standard. The average efficiency increased with age to 10 years, fell off during the transitional period of the dentition, and rose again to reach 66 per cent at age 14. Apparently the masticatory efficiency up to 15 years of age does not become equal to that of the average adult. It seemed likely that studies on an adolescent group would contribute to our knowledge concerning development of masticatory ability from youth to adult. It was the purpose of the present study to obtain this information.

EXPERIMENTAL PROCEDURE

Two hundred fifty students of the Somerville, Massachusetts, High School, ranging in age from 15 to 18 years, were selected for this study. Among these there were, respectively, 53, 90, 72, and 34 adolescents at ages 15, 16, 17, and 18 years. The male athletes within these groups were identified in order to determine whether their masticatory abilities were different from the standard group.

Only masticatory performance on the preferred side with peanuts and the food platform area were recorded. Previous findings had indicated that masticatory efficiency was a suitable general index of masticatory function.[2] Performance tests with carrots, swallowing threshold tests, and observations on the nonpreferred side were eliminated to save time, and this decision was justified because of the high correlation between these and the masticatory performance test with peanuts. In previous work, the maximum biting force was found to have little relationship to masticatory efficiency and food platform area, and in view of this, it was omitted from the testing program.

A 3 Gm. portion of peanuts was chewed for 20 masticatory strokes on the preferred side in order to determine masticatory efficiency. This procedure was repeated 3 times. The preferred side, selected by the patient, is usually the better and most practiced side.[1, 3] Duplicate wax readings were taken on the preferred side in order to determine the food platform area.[4]

RESULTS

In this study the same independent variables were considered for the determination of masticatory efficiency, as previously reported for children.[1]

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These variables included sex, age, and the number and types of teeth in occlusion. Malocclusion could not be considered as a variable in this study, because there were insufficient cases of this condition.

Two comparisons were made between the average masticatory efficiency of males and females. The first comparison was between males and females with all teeth present, and the second was between the sexes using the entire group of subjects. This latter group would contain many subjects with missing teeth. There were no statistical differences in the mean performances of the males and females by either comparison. This result was similar to that found among the younger age groups,[1] and there was the same suggestion from the averages that males might be slightly higher in performance than females.

The subjects with all teeth present were next divided into age groups, in an effort to determine at what age the adult level of efficiency is reached. These cases included a complete dentition exclusive of the third molar, which has been employed as the "standard" dentition previously. The mean efficiencies from age 15 to 18 were 80, 74, 90, and 82, respectively. The increases are irregular because of unavoidable sampling errors, and the drops from 15 to 16 and from 17 to 18 are attributable to chance only. Perhaps the best generalization is that the mean efficiency is 77 at 15½ years, and 86 at age 17½ years. At this latter age the chewing ability is very similar to the 90 per cent which was attained by young adults who were tested at Great Lakes. These new findings allow us to generalize concerning the increase in chewing ability from school to adulthood (Fig. 1). Chewing efficiency starts at 35 per cent for the 6-year-old child with unerupted first permanent molars. When the first permanent molar erupts there is no immediate precipitous increase in chewing ability. There is a gradual improvement in chewing ability over a period of 3 years which reaches a maximum at age 10. Then a retrogression occurs. The transition in the dentition, loss of the primary molars, and substitution by premolars which may not attain perfect occlusion immediately—these factors may be reasons for interruption in the upward trend of chewing ability. From age 11 the chewing ability rises gradually once again over a period of 3 or more years, from approximately 50 per cent to about 85 per cent at age 17 or 18. The increase from age 13 to 18 comes about without change in the number of teeth in occlusion. It has been pointed out[5] that the numbers of teeth in occlusion are not a good index of chewing ability of adults, and this is true also for adolescent children.

The extent of muscular training does not seem to be related to chewing ability. From this group of 250 cases there were 25 subjects, aged 15 to 18, who were members of a team either in football, baseball, basketball, or track. These subjects averaged 75 per cent in efficiency. This finding gives no support to the hypothesis that the masticatory efficiency of athletes is superior to that of a group of subjects who do not receive special athletic training.

The general effect of missing molars was evaluated by averaging the efficiencies of the 47 patients, each of whom had a single molar missing. This group included patients with either first or second permanent molars missing

from maxilla or mandible. This degree of tooth loss reduced the average efficiency to 37 per cent, or one-half. In 29 cases in which 2 nonopposing molars were missing, the loss in efficiency was even greater, and amounted to 79 per cent. Missing teeth generally predispose to lower chewing ability when large groups of people are compared. It appears that missing molars affect masticatory ability enough to justify replacement with functional prosthetic appliances.

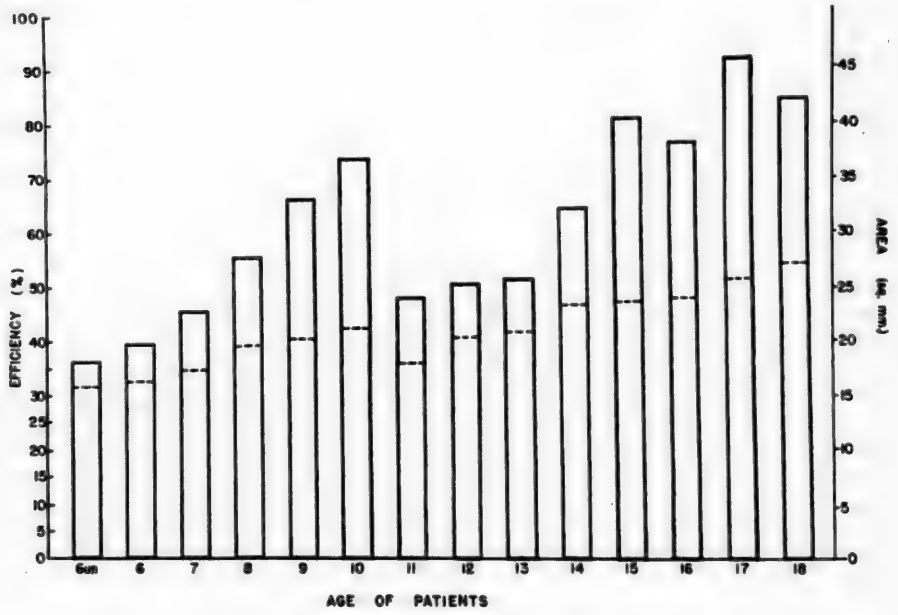

Fig. 1.

The correlation between food platform area and masticatory performance among children is moderately high (0.567),[1] but not as great as that found among adults (0.84).[4] As performance increases with age, there are corresponding increases in area, as shown in Fig. 1. When efficiency decreases from age 10 to 11, there is also a decrease in area. The area parallels efficiency from age 6 to 18.

The correlation coefficient between performance and area for the adolescent group is 0.684. This correlation is higher than that found for younger children, but not as high as the adult "standard." Although the performance in this group has reached the adult level at age 17, the adult area is not attained. Either the patients do not yet use the proper intercuspal occlusion, or the prominence of cusps of teeth prevents their giving as much measurable food platform area as adults usually have.

SUMMARY

A study has been made of the masticatory ability of 250 adolescent subjects from 15 to 18 years of age. Masticatory performance was determined

with peanuts chewed on the preferred side of the dentition, and converted to masticatory efficiency by a table. The occlusal contact area, found to be well correlated in previous studies with masticatory performance, was used again to measure the surface area available for mastication. There was no statistical difference in the masticatory performances between the sexes. Among children with all teeth present there was a gradual increase in performance with an increase in age, and at age 17 the per cent efficiency was similar to that found for young adults. The generalized muscular training of athletes causes no increase in masticatory ability. Subjects lacking a single molar from the preferred side had on the average about one-half the masticatory efficiency of children with a full complement of teeth. The food platform area was moderately correlated with masticatory performance.

The authors wish to acknowledge the cooperation of Mr. Everett W. Ireland, Superintendent of Somerville Schools, and Mr. Albert H. Giroux, principal of the High School, for making the arrangements and assisting in the scheduling of the testing program. Technical assistance was provided by Dr. Helmi Fogels and Miss Madeleine Esserian. Statistical analysis was done by Miss Mary E. Dreisbach.

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TRANSPLANTATION OF DEVELOPING TEETH

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LOSS of the first permanent molar due to caries in childhood or adolescence often constitutes a major dental disaster. Replacement by fixed bridgework is not practicable until late adolescence, by which time mesial drift of the second molar has usually occurred, along with extrusion of the opposing first molar. Since the third molar is often lost in early adulthood for any one of several reasons, its use as a transplant to replace a valuable tooth lost through caries is amply justified.

A project was inaugurated in July, 1952, to attempt to determine whether living teeth could be successfully transplanted from one site to another in human subjects.

OBJECTIVES

Certain criteria were set up, which, if satisfied, would constitute a successful outcome. These were: (1) good color of transplanted tooth, (2) firmness of anchorage in the alveolar process and gingiva, (3) absence of inflammation of the gingiva, (4) positive response to electrical or thermal pulp tests, (5) proper position in the dental arch, achieving maintenance of space and permitting masticatory function, (6) radiographic evidence of health of transplanted tooth and its investing tissues [(a) evidence of healthy periodontal membrane, (b) evidence of new lamina dura of root sockets and alveolar crest, (c) normal size and appearance of pulp chamber and root canal, and (d) evidence of further growth of root and/or normal root formation], and (7) microscopic proof of vitality of pulps of the transplanted teeth and health of the investing tissues.

PROCEDURE

Nineteen transplants were performed in 18 patients over a period of 9 months. The original technic provided the ultimate in asepsis, namely, the hospital operating room with full drapes, gowns, gloves, and autoclaved instruments. The second environment used was the hospital dental clinic, with full aseptic technic, and the last and most frequently used facility was the oral surgery clinic in the School of Dentistry where clean but not autoclaved linen was employed and no gloves were used. All patients were under prophylactic penicillin therapy for a period of 1 week during the period of the trans-

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Fig. 1.—Case 1. Radiographic appearance of incompletely formed lower right third molar before transplantation.

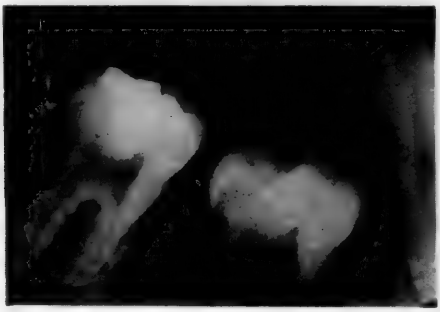

Fig. 2.—Case 1. Appearance immediately after transplantation.

Fig. 3.—Case 1. Two months after transplantation to first molar space. Some eruption and root development have occurred.

Fig. 4.—Case 1. Six months after transplantation. Pulp chamber markedly reduced in size. Distal root formation appears complete, although stunted.

plantation. Since no infections developed and no transplants were lost it was concluded that ordinary oral surgery clinic sterility was entirely satisfactory.

The actual technic varied during the 9-month period both as to the method of taking the tooth from its original position and of placing it in its new location. Originally the method used was that of Apfel,[1] who advocated careful removal of tooth, pericoronal sac, gubernaculum, and a section of overlying mucosa. We found this extremely difficult to accomplish without some tearing of the often delicate sac, but the technic was adhered to in the first 13 cases. The recipient site, in each case the first molar area, was made sufficiently large to permit complete burial of this entire mass, with the small plaque of oral mucosa carefully sutured to the surrounding tissue. Such complete burial required extremely deep bone removal, and considerable concern was felt for the safety of the mandibular canal. In these first cases the entire lingual plate had to be removed before sufficient room could be provided to receive the third molar crown.

TABLE I

SUMMARY ON TOOTH TRANSPLANTATION CASES

PATIENT	AGE (YR.)	SEX	TOOTH	TECHNIC	POSITION IN ARCH	VITALITY POSITIVE (MONTHS)	REDUCTION IN PULP SIZE	LAMINA DURA AND P.D.M. (MONTHS)	ROOT GROWTH (MM.)
L. B.	17	F	$\overline{8}$/	Deep	Fair	10	Marked	6	2.5
L. B.	17	F	/$\overline{8}$	Deep	Fair	10	Marked	11	2.3
R. P.	15	M	$\overline{8}$/	Deep	Good	15	Marked	3	1.5
C. S.	17	M	$\overline{8}$/	Deep	Fair	10	Marked	3	1.3
C. C.	16	F	$\overline{8}$/	Deep	Poor	10	Marked	2	2.3
D. L.	13	M	$\overline{8}$/	Deep	Good	7	Marked	2	0.0
W. J.	14	M	/$\overline{8}$	Deep	Poor	7	Marked	8	0.0
E. B.	19	M	$\overline{8}$/	Deep	Fair	7	Marked	9	0.5
H. H.	17	M	$\overline{8}$/	Deep	Poor	None	Marked	8	1.8
K. G.	15	F	$\overline{8}$/	Deep	Good	4	Marked	7	1.2
C. W.	15	F	$\overline{8}$/	Deep	Good	10	Marked	5	0.0
P. P.	17	M	$\overline{8}$/	Deep	Fair	7	Marked	7	0.0
P. W.	16	M	/$\overline{8}$	Deep	Good	6	Marked	--	2.0
R. C.	18	M	/$\overline{8}$	Splint	Good	--	--	--	--
C. F.	23	M	/$\overline{8}$	Splint	Good	--	--	--	0.0
R. M.	16	M	/$\overline{8}$	Splint	Good	--	Moderate	6	0.0
D. S.	20	M	/$\overline{8}$	Splint	Good	--	Moderate	5	0.0
R. P.	15	M	/$\overline{8}$	Splint	Good	7	Slight	5	1.0
E. H.	15	F	/$\overline{8}$	Splint	Good	3	--	3	0.0

In February, 1953, at the suggestion of Dr. Merle Hale,[2] of the University of Iowa College of Dentistry, we altered the technic markedly. In the last 6 cases the third molar bud was simply elevated from its bed, the pericoronal sac being allowed to tear free during removal. The recipient bed was prepared but did not have to be as deep, and the transplant was inserted in line with adjacent teeth and only slightly below their occlusal plane. An acrylic or cast silver splint was then applied to stabilize the transplanted tooth. The splint was worn for 1 to 4 weeks.

RESULTS

The over-all results are shown in Table I. All transplants but 2 are still in place, and are clinically in a state of health and comfort. Positive vitality tests appeared in 43 per cent of 14 cases tested by the seventh postoperative month. At the tenth month this rose to 85 per cent of 13 cases tested. On

Fig. 5.—Case 2. Decalcified section of one of the transplanted teeth which was extracted because of malposition (magnification ×7; reduced ⅓).

Fig. 6.—Case 3. Decalcified section of the other transplant, removed after one year because of malposition (magnification ×5.2; reduced ⅓).

the other hand, position in the dental arch remained a difficult problem throughout, and the more deeply placed transplants gave by far the greatest degree of trouble on this score. Although clinically healthy and comfortable, 2 were removed 12 months after transplantation as they were so malposed there appeared to be no chance of normal eruption. Each of these extracted transplants was decalcified and prepared for microscopic examination.

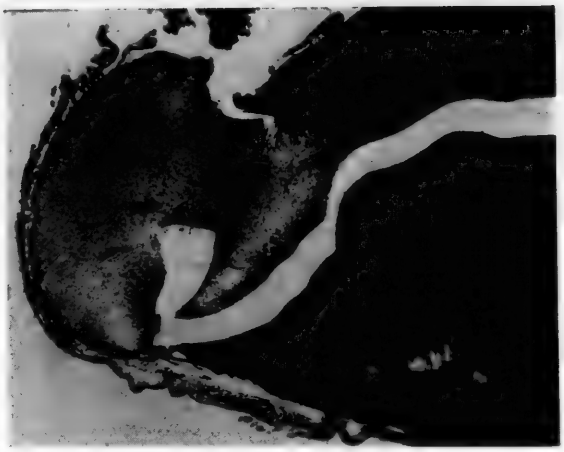

Fig. 7.—Case 3. Higher magnification of root end area. Note vital pulp tissue, irregular cementum, and presence of periodontal membrane fibers (magnification ×30; reduced ⅓).

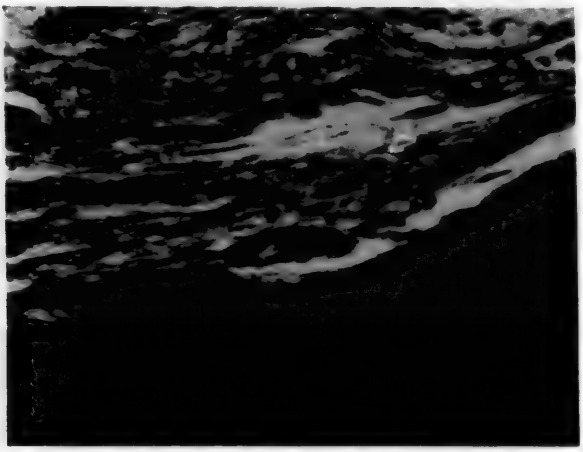

Fig. 8.—Case 3. Higher magnification of root surface. Note large osteoclast in center of field associated with resorption of cementum at this point. Cells of periodontal membrane are arranged longitudinally (magnification ×44; reduced ⅓).

Fig. 9.—Case 3. Normal dentin, irregular dentin, abnormal odontoblasts, and normal pulp are seen (magnification ×30; reduced ⅓).

HISTOLOGIC FINDINGS

Examination of the paraffin sections revealed the following: *First specimen*—Pulp vital. Appears normal except for irregularity of appearance of odontoblasts. Normal dentin (presumably pretransplant) and considerable irregular or secondary dentin (presumably posttransplant) present. A few small pulp stones are seen. *Second specimen*—Pulp vital. Active dentinogenesis at apical end of root. No inflammation of normal pulp tissue noted.

SUMMARY

1. Nineteen lower third molar teeth with incompletely formed roots were transplanted as autografts to lower first molar areas.

2. The criteria of good color and firmness of anchorage in the alveolar process and gingiva have been fulfilled in all cases.

3. None of the transplants has resulted in suppuration or inflammation of the gingiva.

4. Positive response to electrical and thermal pulp tests was achieved in 43 per cent by the fifth month and in 85 per cent by the eighth month.

5. Proper position in the dental arch was not secured in any instance where the transplant was buried deeply. Greatly improved position resulted when the placement was about 1.0 mm. below the occlusal plane of the adjacent teeth. Minor improvements on this score are yet to be developed by orthodontic means or greater precision in splinting.

6. Healthy periodontal membrane and lamina dura have been secured in all cases by the fifth to eighth month, judging from radiographic studies.

7. Pulp chambers consistently showed a tendency to become greatly reduced in size. The significance of this occurrence is not known.

8. Root growth was minimal. Apices either closed off without further growth or remained open with little or no further growth.

9. Microscopic study revealed vital pulps in 2 transplants which were extracted.

CONCLUSIONS

1. Transplantation of incompletely formed lower third molar teeth is a practicable and relatively uncomplicated dental operation.

2. In securing the graft there appears to be no advantage in retaining the pericoronal sac and gubernaculum.

3. Complete burial of the transplant requires a very deep, broad bed and appears to offer no advantage over placement just short of the occlusal plane.

4. A small, open occlusal acrylic or cast silver splint is very helpful in stabilizing and positioning the transplant during the first 1 to 4 weeks.

5. This series of cases was limited to lower third molar transplants in the interests of uniformity for comparison of data. There would seem to be no reason why upper molars could not be managed in the same manner.

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TRANSPLANTATION OF HUMAN TOOTH GERMS TO LOWER ANIMALS

I. Relation to the Host

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INTRODUCTION

PART of a previous report on the intraocular growth of transplanted tooth germs dealt in a small way with such transfers made from human beings (3 fetuses) to experimental animals.[1] Since that time, scope of this work was broadened considerably by the availability of additional human material (28 embryos and fetuses), suitable for in vivo transplantation. Therefore, added significance can be attached to results obtained in these instances further supporting the original findings. It is the purpose of this communication to present these findings stressing at this time relation of the transplant to the host.

MATERIALS AND METHODS

Technical procedures for eye, brain, and axillary transplants have been previously described.[1, 2, 5] Human embryos and fetuses, obtained under aseptic conditions from hospital operating rooms, were kept in a refrigerator at 38° F. until utilized. Preferred human embryos and fetuses were those obtained while still within unruptured amniotic sacks. Usually 2 to 14 hours elapsed before these embryonic tissues were used.

The entire maxilla and mandible were carefully dissected away from each embryo or fetus and placed in sterile, covered Petri dishes. Blood and debris were removed. Tooth germs, exposed by a median incision on the crest of each ridge, were then transferred in a particular order to sterile Petri dishes. Tissues were always kept moist with a sterile normal saline solution. When necessary, a dissecting microscope aided in obtaining material.

Age of embryos and fetuses ranged from approximately 8 to 21 weeks. It was difficult to determine grossly in embryos as young as 8 weeks just what tissues were the anlagen of the teeth. In such cases, block sections of the jaws were transplanted after removal of the oral epithelium. Jaw sections from embryos of a similar age, but not suitable for transplantation, were studied to determine exact locations of the anlagen of teeth. A section of maxilla or mandible with tooth germs in situ was fixed, sectioned, and stained as a control. In other cases where development was advanced to an extent that individual tooth germs could be removed from their follicles, certain ones were

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also fixed, sectioned, and stained as controls. It is not the purpose in this presentation, however, to stress development; but to emphasize relation to the host.

Ages of embryos or fetuses were determined from the mother's statement as to the date of her last menstruation period. Possibilities of errors or inaccuracies had to be considered, but in most cases such information was assumed to be correct. Although crown-rump measurements and weights were recorded, these were not considered relevant to the actual stage or degree of tooth germ development.

Sites used in experimental animals for receiving these transplants were eyes and brains of guinea pigs and axillae of dba mice. In a few instances anterior eye chambers of Australian white giant rabbits were also used. Hosts were carefully selected from stock animals and only healthy young adults of either sex were used. No special care or feeding was instituted following transplantation procedures. When possible, males and females were housed separately. Animals were sacrificed intermittently up to about 60 days and transplants were recovered. Occasionally some transplants were allowed to remain in place for longer periods. Adjacent or attached tissue was generally retained when transfers were recovered to demonstrate relationship of hosts to these transplants. All recovered transfers, after gross examination, were fixed, decalcified, sectioned, and stained for histologic study. Each figure presented, unless otherwise specified, represents a passage from a different subject.

RESULTS

Explants of human tooth germs to anterior eye chambers in guinea pigs and rabbits and to axillae in dba mice were usually accepted by these hosts. Substantiation of this was seen initially in vascularization and stromatization of these transplants. Following this, where there was organization of ameloblasts and odontoblasts, pre-enamel and predentin were laid down. This was followed by mineralization and maturation. Intracerebral transfers were not successful, for at this site there was complete and rapid resorption. Animals killed at intervals, following brain transplantation, failed to reveal any of the explanted tissues. In these instances, the trocar's path was traced to the transplantation site, where all that was usually found were masses of free red blood cells. Infrequently bits of osteoid tissue were found at this transplantation area.

Occasionally none of the transfers from a particular embryo or fetus survived transplantation. This possibly was due to two different causes. Either a vigorous inflammatory reaction took place immediately, or they remained static and gradually resorbed, leaving no trace of the original transfer. These conditions were followed with anterior chamber transplants. Results are depicted in the following figures, which are chronologically arranged.

Fig. 1 is of a section of a transplant recovered from the anterior eye chamber of a female guinea pig 15 days after transplantation. Here, organization of ameloblasts and odontoblasts is evident. Some oral epithelium is attached to the outer epithelial layers of this transplant. There are connective

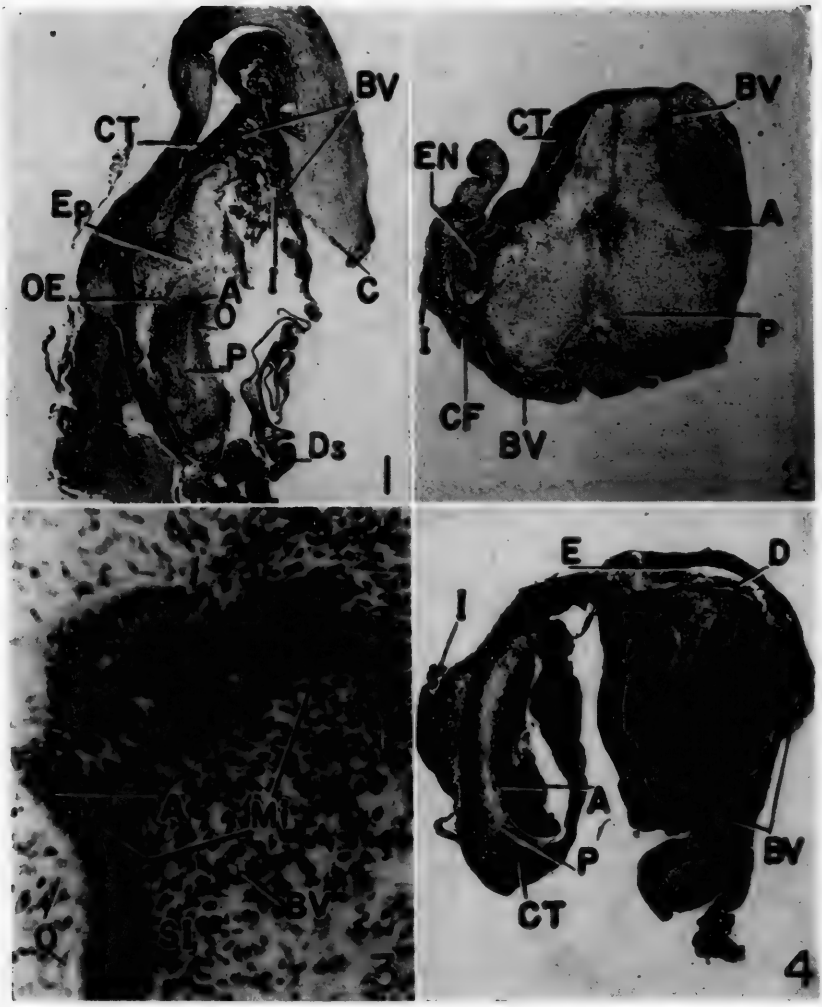

Fig. 1.—Recovered transplant from anterior chamber of female guinea pig, 15 days after transplantation. Original magnification ×35; reduced 3/10.

Fig. 2.—Recovered transplant from anterior chamber of male guinea pig, 16 days after transplantation. Original magnification ×35; reduced 3/10.

Fig. 3.—Higher magnification of Fig. 2 originally at ×475; reduced 3/10.

Fig. 4.—Recovered transplant from axilla of dba Bar Harbor male mouse, 20 days after transplantation. Original magnification ×35; reduced 3/10.

Key to Symbols in Figures

A—Ameloblasts, B V—Blood vessel, C—Cornea, C B—Ciliary body, C F—Collagenous fibers, C T—Connective tissue, Cy—Cystic structure, D—Dentin, Ds—Descemet's membrane, E—Enamel, E N—Epithelial nest of cells, E P—Enamel pulp, Ep—Oral epithelium, Er—Erythrocytes, Fi—Fibroblasts, I—Iris pigment layer, K—Keratin, L—Lacuna, Ly—Lymphocytes, M—Muscle, Mi—Mitotic figure, O—Odontoblasts, O E—Outer enamel epithelium, Os—Osteoid or osteodentin, P—Pulp, R C—Rods and cones, Re—Resorption

tissue attachments between this transfer and the pigmented layers of the iris. Blood vessels in these areas appear viable and are filled with red blood cells. Some are fairly large but none are hyperemic. Between the cornea and the transplant a growth of new connective tissue is observed. However, this transplant is not yet entirely surrounded. A number of thick-walled, small blood vessels are seen in this connective tissue and thinner-walled ones within the pulpal area of the explant. Among arrangements of ameloblasts, as seen in this figure, numerous mitotic figures are evident. Against the dentino-enamel junction a small amount of pre-enamel and predentin has been laid down but no calcified tooth structure has formed. Here we see the survival of the transplant and a beginning of odontogenesis. Morphology of this transplant was disrupted during the transplantation procedures. This tooth germ was an upper deciduous molar obtained from a 4½-month human fetus.

Figs. 2 and 3 represent a recovered human tooth germ transplant originally obtained from a 4½-month human fetus. It remained at the transplantation site in the anterior eye chamber of a male guinea pig for 16 days. These figures are shown because of many mitotic figures in epithelial portions of this transplant. The pulpal area remains large and characteristic. No pre-enamel, predentin, or calcified dental structures are seen. Young connective tissue, incorporated in which are pigmented cells of the iris, surrounds the transplant. Bundles of collagen fibers are also observed. Blood vessels with red blood cells are seen in this connective tissue. It is interesting to note that epithelial remnants, presumably of outer enamel epithelium, have formed nests and cords of cells in various places along the periphery of the pulpal area. In one portion remnants of the ameloblastic cell layer are seen. Many mitotic figures are present, appearing more among cells of the stratum intermedium and not the ameloblasts. These latter cells seem disorganized and shortened with much less cytoplasm than normal. Opposite them odontoblasts, in partial organization, are arranged along the periphery of the pulp. This portion of the recovered transplant is seen in Fig. 3, which is a higher power of Fig. 2.

Figs. 4, 5, and 6 show a recovered axillary transplant from a dba male mouse after 20 days. Origin of this transplant was a deciduous molar tooth germ from a 5-month human fetus. In the process of transplantation the tooth germ layers appear to have been disoriented. This is brought out by the fact that young enamel and dentin appear in one part and in another area a row of ameloblasts is observed, adjacent to remains of the dental pulp. In places epithelial cells have formed clusters, sometimes in a semipalisaded arrangement. Under higher power of this section (Figs. 5 and 6) an ingrowth of young connective tissue appears to have obliterated most of the pulpal area's structure. Bundles of collagen fibers are also seen here. A number of blood vessels with thick walls are observed throughout this part of the section. While Fig. 4 gives an over-all picture of the entire section, Figs. 5 and 6 show in more detail how the pulpal area has been overrun by connective tissue. Ameloblasts have become somewhat disorganized and shortened.

Fig. 7 presents a transplant from a 5-month human fetus, recovered after 25 days in the anterior eye chamber of a male rabbit. A large area of bonelike tissue and remnants of the pulpal area can be seen in places. Vascularization is very good, as evidenced by the large number of vessels filled with red blood cells in the pigmented iris layer adjacent to the transfer. No traces of ameloblasts are found and very few odontoblasts are seen in the pulpal remains.

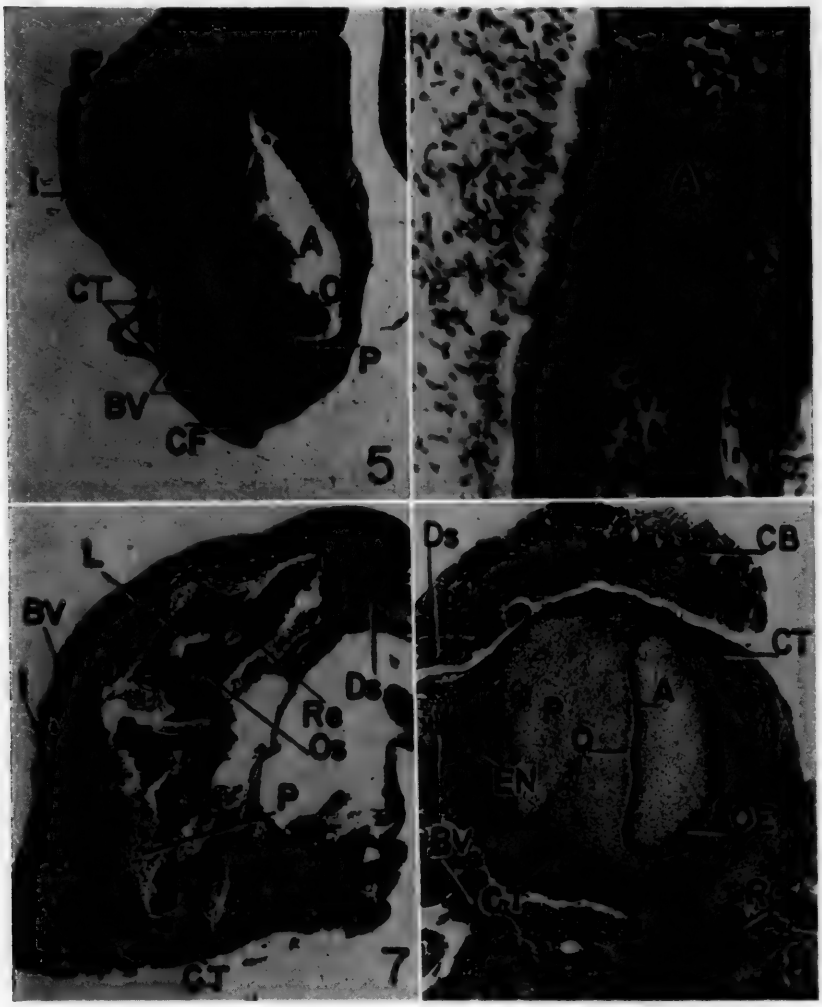

Fig. 5.—Higher magnification of Fig. 4 originally at ×57; reduced 3/10.

Fig. 6.—Higher magnification of Fig. 4 originally at ×475; reduced 3/10.

Fig. 7.—Recovered transplant from anterior chamber of male rabbit 25 days following transplantation. Original magnification ×45; reduced 3/10.

Fig. 8.—Recovered transplant from anterior chamber of male guinea pig, 25 days after transplantation. Original magnification ×45; reduced 3/10.

Presumably, osteoid tissue present represents remnants of the original transplant. Incorporated in this osteoid tissue are structures having a close resemblance to dentin. These small tubules, cut at right angles, contain a dark-staining structure within each of them. These are dentinal tubules holding the protoplasmic processes of odontoblasts. In places, there is osteoclastic resorption and the formation of lacunae. Significance of this revision has been previously discussed.[4]

In Fig. 8 the tooth germ transplant shown has retained its morphologic appearance and shows a number of things. First, there is a large amount of well-vascularized connective tissue, surrounding the whole transplant, in which are bundles of collagen fibers. The location of this transplant can be identified by the presence of Descemet's membrane and parts of the ciliary body. Pigmented layers of the iris as well as rods and cones are seen. It is interesting to observe that islands of epithelial cells, presumably from the outer enamel epithelium, are located in places along the periphery of the transplant. The pulpal area has remained large and the outer enamel epithelium is also seen, as well as the enamel pulp. Organization of odontoblasts is evident in the dental pulp area. Histologically this transfer appears to be at that stage just prior to the laying down of the pre-enamel and predentin matrices. In places along the periphery of the pulpal area of the transplant there is a beginning of an ingrowth of fibroblasts. This transplant demonstrates relationship to the host showing good vascularization. Numerous blood vessels are also shown in the pulpal area. This tooth germ, a lower right cuspid, was obtained from a 21-week fetus and transplanted to a male guinea pig. Fig. 9 is a higher power, to show in more detail an island of epithelial cells along the periphery of this transplant.

Figs. 10 and 11 represent different levels of serial sections of a deciduous lower canine human tooth germ from a 4-month fetus transplanted to the anterior eye chamber of a male guinea pig. This structure was allowed to remain in place for 51 days. Calcified enamel and dentin represent development of mature tooth structures at this site. In relation to the rest of the tooth structure the pulpal area has remained in position. In Fig. 11 organization of odontoblasts and ameloblasts can be more easily seen, as well as connection of this transplant to the iris. There is very little invasion of the pulpal area with fibroblasts. Numerous blood vessels are seen throughout the pulp and in the connective tissue capsule surrounding the whole transplant. In this section survival of the pulpal area and formation of calcified tooth structures are observed.

Figs. 12 and 13 show growth of a human tooth germ from a 4-month fetus after 17 days in the anterior eye chamber of a female guinea pig. These figures also show relation of the transfer to the host as seen by its attachment to the iris. The enamel pulp as well as the dental pulp are seen. The entire explant has been surrounded by a connective tissue capsule in which there are numerous thick-walled blood vessels. In Fig. 13, a higher power of Fig. 12, more detail of the transplant's relationship to the host can be seen. No invasion of the actual transplant by connective tissue is observed.

Figs. 14 and 15 show growth of a deciduous second incisor tooth germ from a 4-month human fetus transplanted to the anterior chamber of a male guinea pig and allowed to remain in place for 30 days. Relationship of this transplant to the cornea and pigmented iris layers is very readily seen. This structure is in the anterior chamber, posterior to the cornea, and is connected to the iris both superiorly and inferiorly by a thin band of connective tissue.

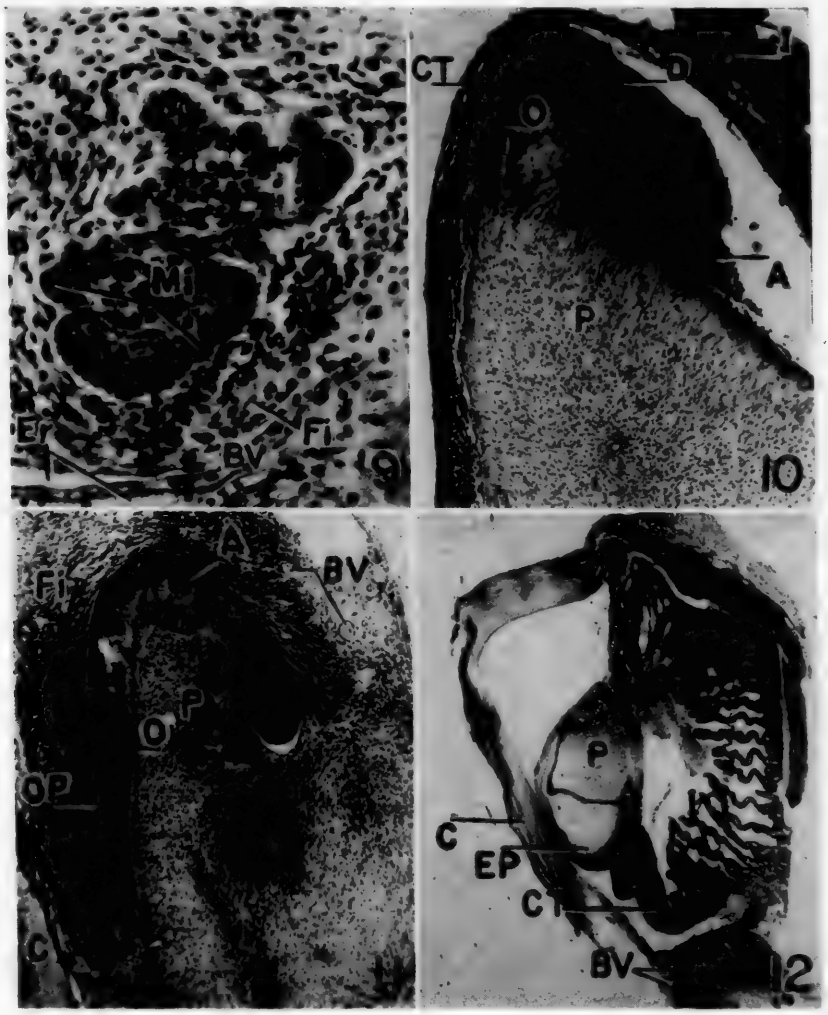

Fig. 9.—Higher magnification of Fig. 8 at × 475; reduced 3/10.

Fig. 10.—Recovered transplant from anterior chamber of male guinea pig, 51 days following transplantation. Original magnification ×145; reduced 3/10.

Fig. 11.—Deeper section of Fig. 10, original magnification ×145; reduced 3/10.

Fig. 12.—Recovered transplant from eye of female guinea pig, 17 days after transplantation. Original magnification ×23; reduced 3/10.

Numerous blood vessels are seen. Fibroblasts are invading and obliterating the pulpal area. Enamel and dentin have formed and have been calcified. Fig. 15 is a higher power of Fig. 14, showing more of the detail of a certain area.

Figs. 16 and 17 represent a deciduous molar tooth germ transplant obtained from a 4½-month human fetus, and recovered after 23 days in the

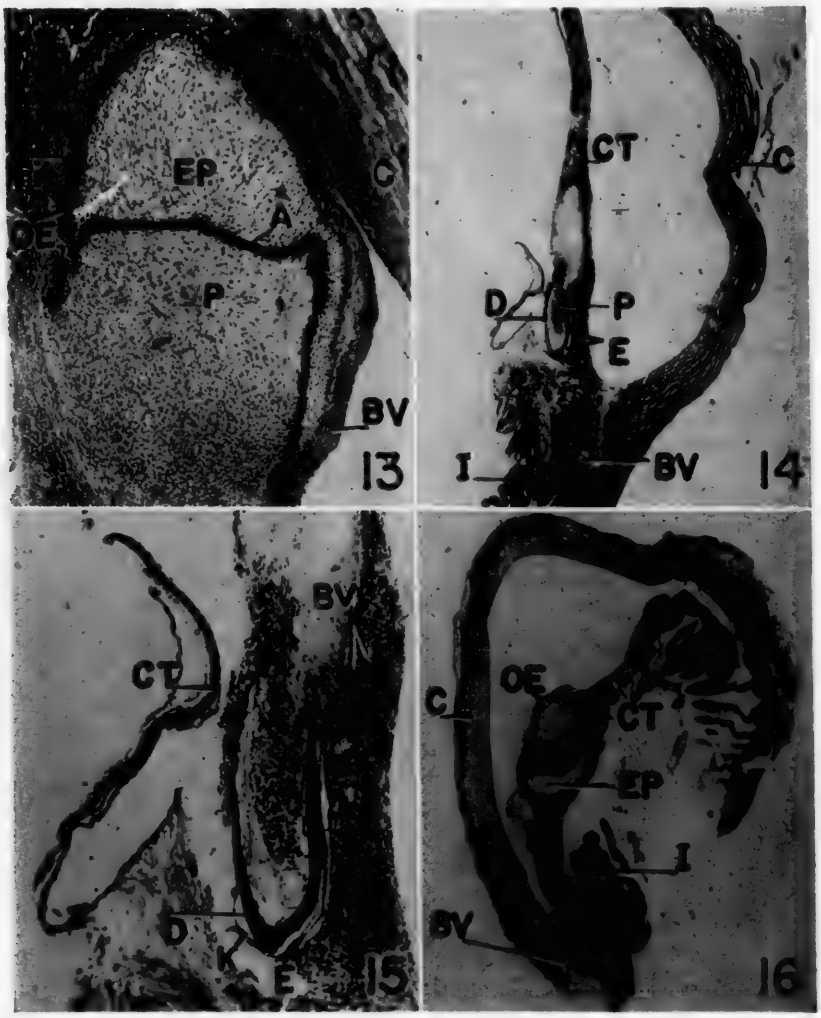

Fig. 13.—Higher magnification of Fig. 12. Originally at ×110; reduced 3/10.

Fig. 14.—Recovered transplant from eye of male guinea pig, 30 days following transplantation. Original magnification ×26; reduced 3/10.

Fig. 15.—Higher magnification of Fig. 14. Originally at ×130; reduced 3/10.

Fig. 16.—Recovered transplant from eye of male guinea pig, 23 days after transplantation. Original magnification ×20; reduced 3/10.

anterior eye chamber of a male guinea pig. Observation of this developing tooth structure indicates transverse cutting of this section. There is a connective tissue attachment in two places to the iris, which has retained its anatomic position in the eye. The cornea can also be seen. In this case the transplant is surrounded by a wall of young connective tissue containing numerous blood vessels filled with red blood cells. Because of the manner in which it has been cut, enamel pulp surrounds most of this section. In the

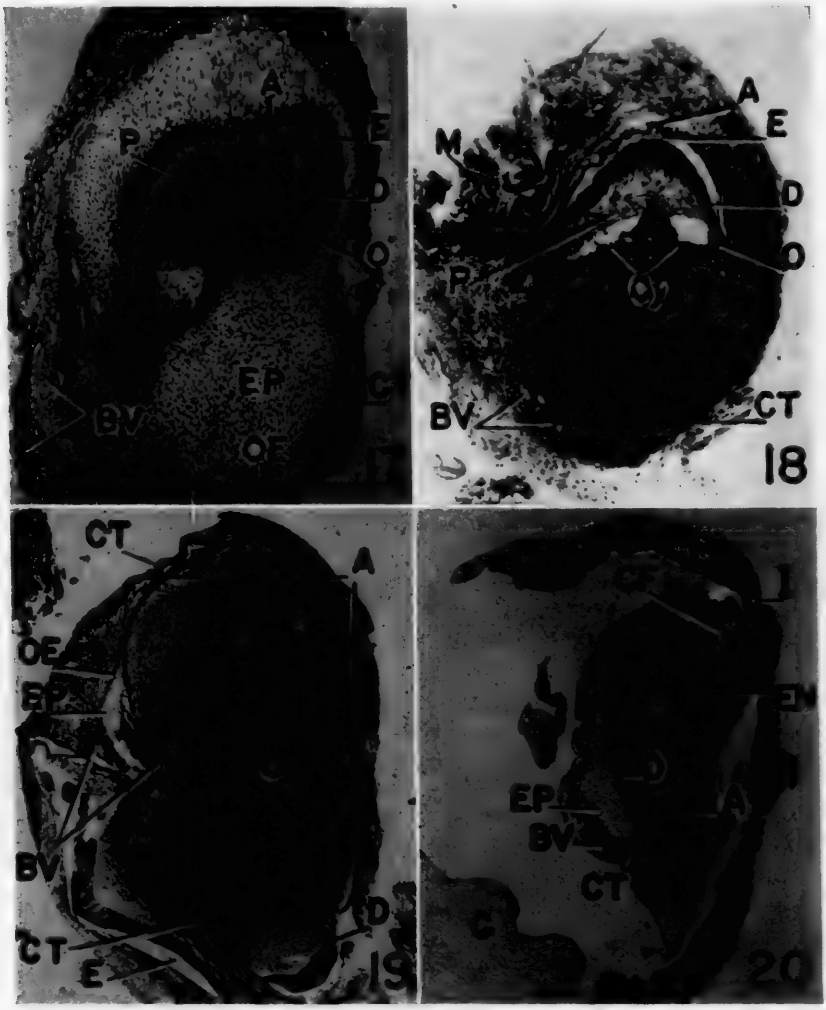

Fig. 17.—Higher magnification of Fig. 16. Originally at ×130; reduced 3/10.

Fig. 18.—Recovered transplant from axilla of dba Bar Harbor male mouse, 28 days after transplantation. Original magnification ×120; reduced 3/10.

Fig. 19.—Recovered transplant from anterior chamber of male guinea pig after 41 days of transplantation. Original magnification ×52; reduced 3/10.

Fig. 20.—Recovered transplant after 49 days in eye of male guinea pig, original magnification ×45; reduced 3/10.

center area where ameloblasts and odontoblasts are in normal relation to each other, enamel and dentin are being formed. Within the pulpal area of this transplant are numerous capillaries. No infiltration of the actual transplant by connective tissue is observed. In one area epithelial cells have begun to form cords or chains, and their position would indicate that they may have come from the outer enamel epithelium and the stratum intermedium. Fig. 17 is a higher power of the tooth structure, showing in more detail the actual transplant.

Fig. 18 represents the recovered transplant of a deciduous canine tooth germ from a 4½-month human fetus after 28 days in the axilla of a dba male mouse. Here formation of mature enamel and dentin are seen. Some of the pulpal area remains, in which there appear several large cystic areas lined with endothelial cells. Directly in the center a sizable invasion of the pulpal area with young connective tissue has begun. Interspersed among this connective tissue are bundles of collagen fibers. This whole transplant appears to be almost entirely surrounded with layers of stratified connective tissue. Bundles of muscle fibers can be seen. Very few odontoblasts remain in position in the pupal area.

Fig. 19 represents a transplant of a deciduous canine from a 4½-month human fetus recovered after 41 days in the anterior chamber of a male guinea pig. In this section enamel and dentin, and ameloblasts and odontoblasts are all in a normal relation to each other. Presumably the transplant as a whole has been disorganized during the transplantation procedures. Connective tissue arising from areas in the pigmented layer of the iris has invaded the pulpal area at a break in the cusp tip. The pulpal area remains large and contains a number of thin-walled blood vessels. In one area there is a focal infiltration of small round cells. Numerous blood vessels are seen in the connective tissue capsule around this transplant.

Fig. 20 shows a deciduous canine tooth germ recovered after 49 days in the anterior eye chamber of a male guinea pig. Source of this explant was a human embryo of 8 weeks. Attachment of this transplant to the pigmented layer of the iris is seen. The entire structure is almost completely surrounded by a stratified layer of connective tissue. Epithelial rests are observed in the area from which the diaphragm forms. Other epithelial cells are in cords and comingled with collagen fibers and fibroblasts at the site of attachment to the iris. Numerous blood vessels are seen in this region, as well as a number of thin-walled vessels in the pulpal area. Ameloblasts are organized, but odontoblasts do not show much organization. Some connective tissue has crowded out most of the enamel pulp.

Of the 28 human embryos or fetuses, tooth germs or anlagen of teeth did not show growth and development in 7 cases. In 5 of these instances the tissues were either nosterile or nonviable, and in the remaining 2, growth of transplants showed nothing but connective tissue and a few thin spicules of bone.

It also appears that where most satisfactory results were obtained, the tissues were obtained from 4- to 5-month fetuses. As observed from these findings, material from extremely young human sources, although accepted, does not survive as well as that obtained within the previously mentioned age ranges. In addition, rate of development, within the limits studied, seemed proportional to the age, for this was slower when younger material was used.

DISCUSSION

It has been the object of this presentation to show that human tooth germs will survive transfer, actually becoming part of the new host. These heterologous transfers may initially survive as tissue cultures, but soon after they can become vascularized. The host's initial response may have been antigenic, but establishment of a blood supply eliminated these transfers' vulnerability to such attacks. It was previously pointed out, in experiments with crystalline cortisone acetate, that the initial lymphogenic reaction by the host could be delayed.[3] However, ultimate fate of such transfers depended upon their stromatization and vascularization. What happened during the first 4 to 5 days following transplantation critically affected future development of a transplant. Furthermore, it has been frequently observed, in the present and in other studies, that transfers may remain static without any reaction favorably or unfavorably on the part of the host. In these instances, transfers were not accepted and the hosts did not evince an antigenic reaction.

With heterologous transplantation of other embryonic organs, functional tests have been used to show their relation to the hosts. In our laboratories various embryonic organs have been heterologously transplanted. Adrenals from rabbit embryos have been transferred to the anterior eye chambers of adrenalectomized guinea pigs.[7] These animals functioned normally until their eyes with the adrenal transplants were removed. Following this removal they died. In another test of functions it has been shown experimentally that heterologous transplants of embryonic kidneys become functional to the extent that urine is secreted by them.[6]

In the present instances evidence of function was seen anatomically as manifested by connective tissue attachments and vascularization, thus making them a part of the animal to which they were transferred. In addition, mature enamel and dentin were formed. Why transfers to the brains of animals refused to grow and were rapidly absorbed presents several questions. It is true that heterologous transfers of tooth germs between experimental animals will survive and show growth and development at this site. In the present instances it may have been that tissues transplanted were either not viable or were infected. This may have been a coincidence each time such transfers were made. Because of the difficulty in obtaining human material in good condition and suitable for transplantation when available, such tissues were usually transplanted to sites where they appeared to survive best.

SUMMARY

1. Transplants of human tooth germs were usually accepted by the hosts in the anterior chamber of the eye and the axilla.

2. Stromatization and vascularization of transplants showed this relation to the hosts.

3. Connective tissue capsules gradually surrounded the whole transplant.

4. Pulpal areas were always invaded with connective tissue when they were disrupted, or they did not retain their anatomic relationship to the rest of the transplant.

5. Cords and clusters of epithelial cells formed, usually from the outer enamel epithelial layer and stratum intermedium.

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THE EFFECT OF SODIUM FLUORIDE AND SODIUM OXALATE ON THE CHICK EMBRYO HEART AND KIDNEY IN TISSUE CULTURE

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THE present experiments were conducted to evaluate the relationship between the effect of sodium fluoride and sodium oxalate on heart and kidney tissue of the 8- to 10-day-old chick embryo in vitro. We attempted to ascertain which concentrations of these ions, expressed in milliequivalents, injure and which do not injure these tissues. A modified procedure to study the effect of sodium fluoride and sodium oxalate on tissue in vitro, wherein tissue size, compound concentration and volume, exposure time, and temperature were controlled,[9, 15] was employed in this series of experiments. For the data reported here, sodium oxalate was selected for comparison with sodium fluoride on the basis of a possible similarity of action in immobilization of calcium and magnesium ions.

Although sodium fluoride and sodium oxalate are implicated as enzyme inhibitors, these effects will not be examined in this report.

Information on the effect of sodium fluoride and sodium oxalate on living tissue in vitro is meager. Loeb[10] induced muscle tetany by subjecting exposed frog nerve and muscle to sodium fluoride and sodium oxalate. The tissue culture experiments of Paff and Boyd[12] show that sodium fluoride inhibits tissue growth of growing chick embryo bone at a concentration of 1:200M (5.0 meq. per liter of F^-, or 210 ppm NaF*) but has no effect at a concentration of 1:500M NaF (2.0 meq. per liter of F^- or 84 ppm NaF*). Sodium fluoride decreases the rate of rachitic cartilage calcification in vitro,[14] and when magnesium is added, such inhibitory effects progress beyond the initial stage.[6]

Pollack[13] microinjected sodium oxalate into the cytoplasm of amoebae, and a calcium oxalate precipitate occurred with a positive reaction to alizarin red, the amoebae recovering 3 hours later. If the amoebae which were previously injected with sodium oxalate were again injected with calcium chloride, the recovery time was decreased to less than 1 hour, indicating that calcium in the cytoplasm was again replenished.

METHODS AND MATERIALS

The tissues used in these experiments were heart and kidney of the chick embryo, chosen because they presented the opportunity to observe not only growth but function as well. The test solutions were prepared from stock solution of sodium fluoride or sodium oxalate in sterile double distilled water (containing 0.5 per cent sodium chloride), by dilution with Tyrode's solution minus Ca and Mg at pH 7.2 to 7.4. The stock solutions were kept at room temperature in

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*Author's calculations.

glass-stoppered Pyrex bottles and discarded after 1 week. The sodium oxalate stock solution was heated in a 56° C. water bath for one-half hour to minimize bacterial contamination; sodium fluoride stock solutions were not heated. All dilutions were prepared aseptically the same day the tissues were prepared.

The heart ventricles and the kidneys of 8- to 10-day-old chick embryos were removed aseptically, immediately placed in Petri dishes containing Tyrode's solution, and cut to approximately 1.0 cubic millimeter pieces. The fragments were then immersed in 1 ml. of sodium fluoride or sodium oxalate of known concentration in Ca- and Mg-free Tyrode's solution in large depression slides in covered Petri dishes. The tissue fragments (not exceeding ten pieces) were left in the test solutions for 1 hour at room temperature. At the end of 1 hour, as much as possible of the test fluid was removed and replaced by Tyrode's solution containing Ca and Mg. Six to 8 drops of horse serum and chick embryo extract mixture (1:1) were added in an attempt to immobilize the remaining fluoride or oxalate ions. The tissues were then explanted into tissue culture media containing 2 drops of fowl plasma nutrient mixture (horse serum, chick embryo extract, Tyrode's solution, and heparinized fowl plasma, 2:1:1:1), sealed in Maximow's double cover slip depression slides, and left to clot.[1] It is to be noted that the amount of sodium fluoride or sodium oxalate that was carried from the test solution to the plasma nutrient medium was never sufficient to prevent the heparinized fowl plasma from clotting. In the kidney cultures sufficient phenol red indicator was added so that the plasma coagulum had a definite red appearance.[2, 3] Within 2 hours the explants were examined for heart muscle contractions or kidney tubule dye concentration, and placed in a 38° C. incubator. The tissue cultures were examined every day for 3 days, and proliferation and migration of cells were observed to reach the maximum on the third day, followed by a gradual liquefaction of the plasma clot. In several series, during an incubation period of 7 to 10 days, the cultures were washed every third day with Tyrode's solution for 20 to 30 minutes, fresh fowl plasma-nutrient mixture was added, and the cultures were again sealed in Maximow's depression slides. It should be stated here that this "washing" of these cultures did not appreciably alter the results from those obtained from unwashed cultures.

The controls without sodium fluoride or sodium oxalate were immersed in Ca- and Mg-free Tyrode's solution for the same time that the experimental tissue fragments were treated. There was no observable difference between the effect on tissue growth of the Ca- and Mg-free Tyrode's solution and the standard Tyrod's solution (containing Ca and Mg).

RESULTS

Criterion of Survival.—When a tissue explant showed peripheral proliferation and migration of cells it was tabulated as a survived explant, but if no growth occurred or only an occasional fibrocyte appeared at the periphery, the explant was considered a negative explant.

Criterion of Function.—The heart tissue explants were considered functionally positive when muscular contractions were detected. Positive phenol red

indicator concentration was recorded if any kidney tubule or tubules of the explant retained the dye.[4] Negative function was recorded if there was no concentration of the dye. In some explants, however, the tubules concentrated but did not retain the indicator, and were considered positive to negative.

Criterion of Injury.—An explant was considered slightly injured when the periphery of the tissue fragment showed few areas of erosion and some areas of no cell migration. Marked injury was indicated not only when the explant had poor cellular growth but also when loose spheroid cells surrounded the tissue explant. The kidney explants, when markedly injured, had more loose spheroid cells than the heart tissue explants. These loose cells were never observed to change shape or position.

In all heart explants that survived treatment, showing rhythmic contractions, there was a lag phase of 1 to 2 hours after tissue culture implantation, before contractions were observed. In some heart explants the contractions were over-all, while in others, only certain segments contracted. Contraction was observed to continue at a rhythmic rate for several days in both instances.

The first indication of phenol red concentration in cells of the kidney tubules occurred within 1 hour and apparently there was no time lag in recovery, the cells appearing darker than the surrounding medium. When these kidney cultures were incubated 24 to 48 hours, some of the tubules were markedly distended, due to absorption of the dye from the surrounding medium, also indicating a positive dye concentration.[4]

Heart Group—Sodium Fluoride Series (Table I).—Very good growth was observed in all treated explants that survived 50 meq. per liter of F (2,100 ppm sodium fluoride), a gradual decrease in the amount of cell growth occurring with increasing F ion concentration, starting at 59.5 meq. per liter of F (2,500 ppm sodium fluoride). Complete absence of cellular growth was noted in all explants treated in 138 meq. per liter of F (5,800 ppm sodium fluoride). Heart muscle contractions were observed in explants exposed to F in concentrations up to 50 meq. per liter (2,100 ppm sodium fluoride). However, in a few explants, heart contractions were observed, even after the tissue had been immersed in a solution of 100 meq. per liter of F (4,200 ppm sodium fluoride). These instances of contraction do not have quantitative significance because only 20 per cent of untreated explants contracted. When the treated explants were immersed in 59.5 meq. per liter of F (2,500 ppm sodium fluoride), a peripheral disintegration (slight injury) was detected. This injury was marked in explants exposed to 126 meq. per liter of F (5,300 ppm sodium fluoride).

Heart Group—Sodium Oxalate Series (Table II).—Excellent growth was obtained in some of the explants, even those treated at the highest concentration of oxalate used, 373 meq. per liter (25,000 ppm sodium oxalate). A gradual decrease of cellular proliferation and migration was observed for the majority of explants, starting at a concentration of 179 meq. per liter of oxalate (12,000 ppm sodium oxalate), slight injury occurring at 95.6 meq. per liter of oxalate (6,400 ppm sodium oxalate) and becoming marked in most of the explants at

299 meq. per liter of oxalate (20,000 ppm sodium oxalate). Heart muscle contraction was erratic and one explant contracted at the highest concentration of oxalate tested, 373 meq. per liter (25,000 ppm sodium oxalate).

Kidney Group—Sodium Fluoride Series (Table I).—Excellent growth was observed in all treated explants at the test concentration of 11.9 meq. per liter of F (500 ppm sodium fluoride), while a gradual decrease in the amount of

TABLE I
EFFECT OF CONCENTRATION OF SODIUM FLUORIDE ON CHICK EMBYRO TISSUE EXPLANTS

MEQ./L.* OF F⁻	NaF (PPM)	HEART			KIDNEY		
		#	%	HB	#	%	PR
(Controls)	–	95	100	+	141	100	+
2.38	100	21	100	+	18	100	+
5.95	250	nd	nd	nd	12	100	+
11.9	500	12	100	+	20	100	+
17.9	750	nd	nd	nd	24	100	+
23.8	1,000	51	100	+	58	100	PN
26.2	1,100	15	100	+	15	100	PN
35.7	1,500	15	100	+	32	85	PN
38.1	1,600	15	93	+	12	92	PN
47.6	2,000	52	96	+	58	78	PN
50.0	2,100	9	100	+	28	91	PN
59.5	2,500	35	89	–	24	80	PN
61.9	2,600	9	78	–	11	82	PN
71.4	3,000	51	73	+	58	76	PN
76.2	3,200	8	63	+	12	83	–
83.3	3,500	55	73	–	57	68	–
88.1	3,700	8	50	–	12	67	–
95.2	4,000	61	82	–	54	39	–
100	4,200	18	33	+	12	50	–
107	4,500	31	65	–	34	15	–
112	4,700	8	25	–	12	25	–
119	5,000	37	43	–	56	22	–
126	5,300	8	25	–	12	17	–
131	5,500	40	8	–	42	24	–
138	5,800	8	0	–	12	17	–
143	6,000	12	0	–	28	22	–
150	6,300	8	0	–	12	8	–
155	6,500	18	0	–	16	0	–
176	7,400	nd	nd	nd	12	0	–
200	8,400	8	0	–	12	0	–
226	9,500	8	0	–	12	0	–

*1,000 ppm equals 23.81 meg./L.
+ Functional explants.
– Nonfunctional explants.
Number of explants used.
% Per cent survival (see text).
HB Heart contraction.
PR Phenol red concentration.
PN Phenol red not retained in tubules (see text).
nd Not done.

cellular growth was noted for explants treated with higher concentrations of F, starting at 17.9 meq. per liter (750 ppm sodium fluoride). No growth was observed in any explant exposed to 155 meq. per liter of F (6,500 ppm sodium fluoride). The first evidence of slight injury and surival of the majority (78 per cent) of the treated kidney tissue fragments occurred consistently at 47.6 meq. per liter of F (2,000 ppm sodium fluoride); marked injury and survival of some fragments (39 per cent) were noted at 95.2 meq. per liter of F (4,000

ppm sodium fluoride). Phenol red was concentrated in the tubules after treatment with 71.4 meq. per liter of F (3,000 ppm sodium fluoride), or less. The dye was retained in the proximal tubules after treatment with F, up to 17.9 meq. per liter (750 ppm sodium fluoride).

TABLE II
EFFECT OF CONCENTRATION OF SODIUM OXALATE ON CHICK EMBRYO TISSUE EXPLANTS

MEQ./L.* OF $C_2O_4^-$	$Na_2C_2O_4$ (PPM)	HEART			KIDNEY		
		#	%	HB	#	%	PR
(Controls)	–	81	100	+	124	100	+
11.2	750	nd	nd	nd	12	100	+
12.5	840	8	100	+	8	100	+
22.4	1,500	nd	nd	nd	12	100	+
23.9	1,600	12	100	+	12	100	+
25.4	1,700	16	100	+	20	100	+
34.3	2,300	nd	nd	nd	12	100	+
37.3	2,500	8	100	–	8	100	+
44.7	3,000	12	100	–	12	100	+
47.8	3,200	12	100	+	12	100	+
50.8	3,400	16	100	+	20	100	PN
59.7	4,000	12	100	–	12	100	PN
62.7	4,200	8	100	–	8	100	PN
67.2	4,500	nd	nd	nd	12	100	PN
71.7	4,800	12	100	+	12	100	PN
74.7	5,000	28	100	+	32	97	PN
88.1	5,900	12	100	–	8	100	PN
89.6	6,000	12	100	–	12	92	PN
95.6	6,400	12	92	–	12	100	PN
100	6,700	6	100	–	20	100	PN
105	7,000	36	98	–	27	89	PN
112	7,500	8	88	–	8	100	PN
119	8,000	12	75	–	12	58	–
125	8,400	16	94	+	20	85	–
134	9,000	45	88	–	34	86	–
137	9,200	8	88	–	8	75	–
149	10,000	38	93	–	28	79	–
164	11,000	38	92	–	31	61	–
176	11,800	12	75	–	21	67	–
179	12,000	21	95	+	16	94	–
194	13,000	33	91	–	33	73	–
200	13,400	12	83	–	12	67	–
209	14,000	19	100	–	18	95	–
224	15,000	38	84	–	40	67	+
225	15,100	12	59	–	12	42	–
254	17,000	20	80	–	15	27	–
269	18,000	20	50	–	9	22	–
284	19,000	20	60	–	20	20	–
299	20,000	22	27	–	18	33	+
314	21,000	15	33	–	30	13	–
329	22,000	12	42	–	15	20	–
343	23,000	14	50	–	15	27	+
358	24,000	8	38	–	8	38	–
373	25,000	27	89	+	22	55	+

*1,000 ppm equals 14.93 meq./L.
For explanation of abbreviations, see Table I.

Kidney Group—Sodium Oxalate Series (Table II).—All treated explants showed very good growth at 100 meq. per liter of oxalate (6,700 ppm sodium oxalate). The proliferation and migration of cells started to decrease at 50.8 meq. per liter of oxalate (3,400 ppm sodium oxalate). Excellent growth occurred in many explants even at the highest concentration of oxalate, 373 meq. per

liter (25,000 ppm sodium oxalate). Phenol red concentration in the kidney tubules was consistently positive in explants exposed to oxalate in concentrations ranging from 11.2 to 47.8 meq. per liter (750 to 3,200 ppm sodium oxalate), and only occasional positive phenol red concentration occurred where the test concentration ranged from 224 to 373 meq. per liter of oxalate (15,000 to 25,000 ppm sodium oxalate). Phenol red was not retained by the kidney tubules at test concentrations of 50.8 to 112 meq. per liter of oxalate (3,400 to 7,500 ppm sodium oxalate). There was marked fluctuation in injury for different concentrations. Slight injury was first seen at 105 meq. per liter of oxalate (7,000 ppm sodium oxalate), while marked injury appeared at 284 meq. per liter of oxalate (19,000 ppm sodium oxalate).

Other Observations.—Calcium oxalate crystals were observed in the plasma clots of the heart tissue explants at concentrations as low as 25.4 meq. per liter of oxalate (1,700 ppm sodium oxalate) and of the kidney tissue explants at 22.4 meq. per liter of oxalate (1,500 ppm sodium oxalate). The calcium oxalate crystals observed in the tissue culture clots did not interfere with either cellular growth or function. Many explants grew so well that the cells migrated into the calcium oxalate precipitate and pushed the crystals away from the periphery of the explant.

DISCUSSION

In our previous experiment,[5] we did not replace the test fluid by Tyrode's solution before explanting. Thus some amounts of the test chemicals were being carried with the tissues into the culture media. The results of this method seemed to indicate a similarity of action of the 2 test compounds on a milliequivalent basis.

In the experiments reported here, the tissue fragments were "washed" after exposure to the test solutions.

The results do not indicate a similarity of action of fluoride and oxalate ions on an equivalent basis. The per cent survival of the heart and kidney explants, weighted by the number of explants used at each test concentration, plotted against the average concentration of the test solutions for an increment of 20 meq. per liter of fluoride and for 40 meq. per liter of oxalate (data from Table I and Table II) gives the curves shown in Fig. 1. From these data it is clear that sodium fluoride is about 2.5 times as toxic as sodium oxalate for both tissues, and that the heart tissue is a little less sensitive to both substances. Mobility of the ions, permeability of the cells and diffusion gradients in the cytoplasm, and presence of other ions may drastically affect the equilibrium conditions of the calcium of tissues in various concentrations of the test substances. In regard to competitive cations, magnesium may be critical in concentration balance with calcium.[7] Calcium may counteract the effect of magnesium in living tissues.[11] It is interesting to note that the survival of heart tissue at 150 meq. per liter of F is 0.0 per cent, while no concentration of oxalate tested suppressed the survival below 20 per cent (Fig. 1).

From the present data, we could find no correlation in chemical equivalent effect between sodium fluoride and sodium oxalate on either mesonephron or heart ventricle of 8- to 10-day-old chick embryos. One obvious conclusion is that the immobilization of calcium, per se, is not the limiting factor in the results obtained in this study. We have tested, although not so completely, other normal tissues with the same procedure we are now reporting, and have obtained similar results. The tissues tested were the chick embryo gonads for growth, mouse salivary glands for growth and terminal cluster formation, and the mouse spleen for growth and amoeboid migration.

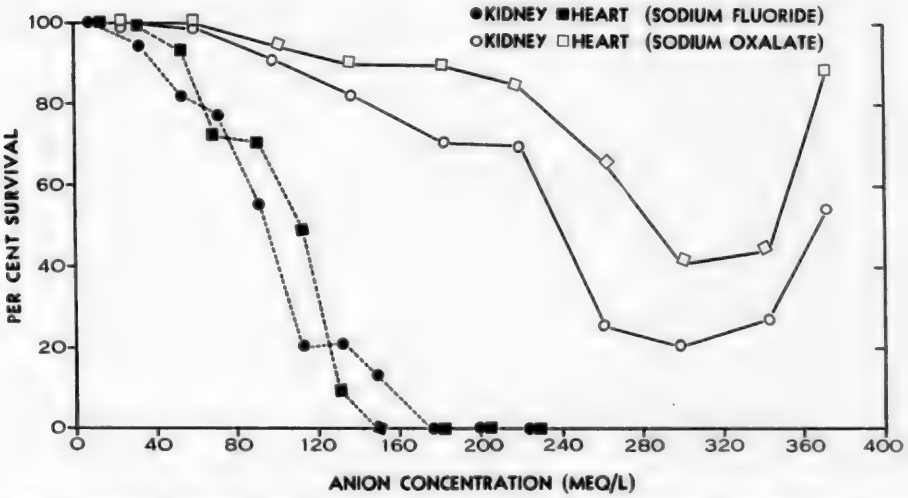

Fig. 1.—Effect of fluoride and oxalate ions on survival of 8- to 10-day-old chick embryo heart and kidney.

The observation of loose spheroid cells at the periphery of the heart, and especially of the kidney explants, may be due to the same phenomenon that Herbst (cited by Heilbrunn[8]) reported fifty years ago, substantiated by many embryologists, that in the absence of calcium, embryonic cells separate from one another. In the kidney explants not only were there many loose spheroid cells at the periphery but also the tubules were somewhat scattered in the plasma coagulum, thus indicating a loss of intercellular cohesiveness in the mesodermal stroma.

Although the chemical equivalent effect could not be correlated in these experiments, the use of milliequivalents of ions per liter should be utilized instead of parts per million (ppm), as the molecular weight of one chemical may be disproportionate with the weight of another chemical, even though they possess similar equivalent chemical reactions. For example, 100 meq. per liter of fluoride and 100 meq. per liter oxalate are equal in chemical equivalency, but the parts per million are not; sodium fluoride is 4,200 ppm and sodium oxalate is 6,700 ppm.

Our approach to an interpretation of these experimental results has been entirely on the hypothesis that fluoride inhibits calcium in tissues, rather than on any action of fluoride on specific enzymes or enzyme systems. The effect of the sodium ions from sodium fluoride or sodium oxalate on the tissue explants or the influence of the sodium ion concentration on fluoride or oxalate toxicity has not been considered in this study, although a difference in toxicity of fluoride and oxalate is expected for a different cation.[16]

SUMMARY AND CONCLUSIONS

1. A method was devised to study the comparative effect of sodium fluoride and sodium oxalate on heart ventricles and kidney mesonephra of 8- to 10-day-old chick embryos under controlled conditions.

2. Sodium fluoride appears to be two and one half times as toxic as sodium oxalate and the heart is less sensitive than the kidney.

3. The chemical equivalent effect could not be correlated with sodium fluoride and sodium oxalate.

4. Calcium and magnesium balance may be critical, but the immobilization of calcium, per se, is not the limiting factor. The inhibition of enzyme actions and the differences of sodium and potassium on tissues, although not discussed, are not ruled out as factors.

5. The loss of intercellular cohesiveness of the explants is demonstrated (Herbst phenomenon).

6. The use of milliequivalents per liter of ions (meq./L.) is advocated instead of parts per million (ppm).

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THE REMOVAL OF FLUORINE FROM DILUTE SOLUTIONS OF SODIUM FLUORIDE AND STANNOUS FLUORIDE BY POWDERED DENTAL ENAMEL

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IT HAS been generally accepted that the mechanism by which fluorine decreases the solubility of dental enamel in acid and partially diminishes subsequent cavity formation is one which makes the surface of the enamel more resistant to acid attack. Initial absorption of fluoride by the enamel might well be followed by ion exchange involving the replacement of hydroxyl, carbonate, or phosphate ions of the enamel by fluoride ions, but this has not been unequivocally experimentally established.

In true absorption, with no special reference to powdered dental enamel, a dynamic equilibrium is established between the absorbent and the absorbate. In general, the more concentrated the absorbate in the medium surrounding the absorbent, the greater will be the amount absorbed. One would expect the absorption of fluoride ions by powdered enamel to reach a maximum when the entire surface becomes covered with a monolayer of fluoride ions since the absorbed ions would then repel other ions of the same charge. If the phenomenon is one of absorption, desorption should occur upon exposure of the powdered enamel to a lower concentration of fluoride ions than that existing when equilibrium was established. There is no experimental evidence in the literature to support the latter premise. The fact that topical protection of enamel against acid solubility does persist for at least 2 years[1, 2] suggests that if absorption is the initial phenomenon, then it must be followed by a secondary chemical reaction.

In the absorption studies by Volker, Hodge, Wilson, and Van Voorhis,[3] radioactive fluoride in solutions of sodium fluoride of different concentrations was stirred with powdered dental enamel for 30 minutes at 40° C.; the enamel was separated, and both the enamel and the solution were analyzed for fluorine by measuring the activity of the radioactive fluoride tracer. The removal of fluorine from the solution by the enamel was reported to follow a Freundlich absorption isotherm.

Straub and Adler[4] measured the change in fluoride concentration of a solution of sodium fluoride which was stirred with coarse, crushed tooth enamel for periods of time up to 200 minutes. The fluoride concentration of the solution reached a minimum after 1 hour and thereafter increased slowly.

Since stannous fluoride and sodium fluoride differ in their ability to reduce powdered enamel solubility,[5, 6] whole teeth solubility,[7] and experimental caries

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in the rat[8-10] and hamster[11] as well as in human beings,[12] the present investigation was undertaken to determine whether or not the source of the fluorine has an effect on the amount of fluorine removed by enamel. Some evidence indicates that enamel removes more fluoride from solutions of stannous fluoride than from sodium fluoride regardless of the age of the solution,[13] and the data found in this experiment corroborate these findings by demonstrating that quantitatively more fluorine is removed from stoichiometrically equivalent solutions of stannous fluoride than from sodium fluoride.

EXPERIMENTAL

Materials.—

Sodium fluoride: This was a spectroscopically pure product of the Aluminum Corporation of America. It was also used as a primary standard in all the fluorine determinations. *Stannous fluoride:* This was prepared according to the method of Nebergall, Muhler, and Day.[14] *Water:* Water redistilled in Pyrex from an alkaline potassium permanganate solution was used. Aliquots from a 1 L. concentrate of 5 L. of this water were found to contain no fluorine upon analysis. *Powdered human tooth enamel:* This was prepared by the method of Manly and Hodge[15] as modified by Muhler, Boyd, and Van Huysen.[5] The enamel passed a 150-mesh Tyler-type screen and was retained by a screen of 200 mesh.

Removal of Fluorine by Enamel From Solutions of Sodium Fluoride.— Solutions of sodium fluoride with concentrations of fluorine varying between 2.00 and 50.0 μg per milliliter were prepared by volumetric dilution of a standard solution of sodium fluoride containing 1,000.0 μg of fluoride per milliliter. Final dilutions were made with a 5 ml. microburet and 100 ml. volumetric flasks. Samples of powdered tooth enamel weighing 0.3000 ± 0.0005 Gm. were shaken with 15.00 ± 0.04 ml. of fluoride solution for six hours in a water bath maintained at 37.00 ± 0.05° C. Next, the liquid and a small quantity of solid were decanted into 15 ml. centrifuge tubes and centrifuged for 15 minutes at 2,200 rpm. The supernatant liquid was decanted and saved for analysis. Duplicate samples were shaken at each concentration. The containers used for shaking and storing the experimental solutions were 60 ml., glass-stoppered, soft-glass bottles, the stoppers of which were sealed with molten paraffin. The final fluoride concentration was determined by the modified Williams titration[16] after distillation by the procedure recommended by Flagg.[17,18] Aliquots of the various solutions were taken for distillation with graduated pipettes in order that the volume of the solution might be adjusted to contain less than 70 μg of fluorine. This procedure ensured against contamination of the stills and thus reduced the possibility of error in subsequent analysis. Points for the preparation of a calibration curve were within a 3 per cent range of the curve, and duplicate analysis of the same experimental solution verified this value as the limit of reliability of the fluorine analysis. Samples prepared in the same manner as the experimental ones but containing no enamel were shaken in the thermostat

for 6- and 24-hour periods; analysis of the solutions showed that no detectable amount of fluorine had been removed from solution by the containers or lost due to handling procedures.

The analytic data are given in Table I. The initial concentration of fluoride, α, was obtained by quantitative dilution of a more concentrated sodium fluoride solution. The final concentration, C, was determined by analysis. The change in concentration (α-C), multiplied by the total volume of the solution, 15 ml., gave the amount of fluoride removed from solution by 0.3000 gram of enamel. This product multiplied by 10/3 gave the quantity of fluoride removed from solution by 1 gram of tooth enamel, ΔF:

$$\Delta F = 15.0\ (\alpha\text{-}C)\ 10/3 \tag{1}$$

The accuracy of ΔF is inversely proportional to the fraction of the total fluorine that is removed from solution. The percentage accuracy is the ratio of the standard 3 per cent error of the analysis and the fraction of fluorine removed from solution:

$$\text{percentage error in } \Delta F = 3\% \frac{(\alpha - C)}{\alpha} \tag{2}$$

At the lowest concentration, approximately one-half the total fluoride was removed from solution; hence the possible error in ΔF was 6 per cent or 3.3 μg of fluorine per gram of enamel. At the highest concentration, only one-tenth of the fluorine was removed from solution and the error was possibly 30 per cent or 69 μg per gram of enamel. All pairs of duplicate samples agreed within this error limit except the pair at the initial fluoride concentration of 25.0 μg per milliliter.

TABLE I

REMOVAL OF FLUORINE BY POWDERED HUMAN DENTAL ENAMEL FROM SOLUTIONS OF SODIUM FLUORIDE

SAMPLE NUMBER	CONCENTRATION OF F IN SAMPLES		F REMOVED (ΔF) (μG/GRAM)	pH OF INITIAL SOLUTION
	INITIAL (α) (μG/ML.)	FINAL (C) (μG/ML.)		
1	2.00	0.95	52.5	7.8
2	2.00	0.96	52.0	7.8
3	5.00	2.7$_7$	11.0$_1$	7.4$_0$
4	5.00	This sample was lost		
5	10.00	7.1$_3$	14.0$_2$	6.9$_8$
6	10.00	7.1$_3$	14.0$_2$	6.9$_8$
7	15.00	11.0$_8$	16.0$_9$	--
8	15.00	12.0$_0$	15.0$_0$	--
9	20.00	15.0$_7$	21.0$_5$	6.8$_7$
10	20.00	16.0$_3$	18.0$_5$	6.8$_7$
11	25.00	20.0$_1$	24.0$_7$	6.7$_7$
12	25.00	21.0$_4$	18.0$_9$	6.7$_7$
13	30.00	25.0$_9$	20.0$_5$	6.6$_9$
14	30.00	25.0$_9$	20.0$_5$	6.6$_9$
15	40.00	35.0$_2$	23.0$_5$	--
16	40.00	35.0$_3$	23.0$_5$	--
17	50.00	45.0$_4$	22.0$_9$	6.4$_4$
18	50.00	45.0$_3$	23.0$_5$	6.4$_4$

On Fig. 1, ΔF is plotted as a function of C. Since the slope of the curve decreases with increasing fluoride concentration, the phenomenon occurring appears to be one of absorption. However, later studies regarding the rate of

removal of fluoride from solution by enamel showed that equilibrium had not been established even after 6 hours' shaking of enamel with fluoride solutions. Thus, these data fail to corroborate the findings of Straub and Adler.[4] Since all absorption isotherms are necessarily based upon the assumption that equilibrium has been established, it is not surprising that neither plots of log F versus log C nor plots of C/F versus C gave straight lines.

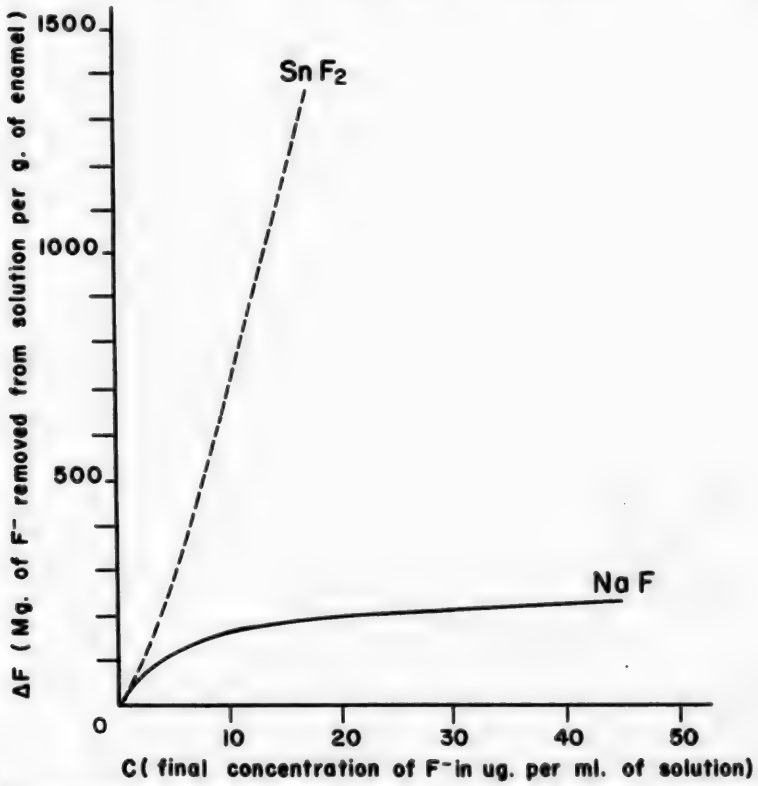

Fig. 1.—Removal of fluorine by powdered dental enamel from solutions of sodium fluoride and stannous fluoride.

Removal of Fluorine by Enamel From Solutions of Stannous Fluoride.—The procedure used for the stannous fluoride solutions was essentially the same as that employed for the sodium fluoride solutions except that the stannous tin solutions were protected against atmospheric oxidation and the fluoride concentration of the solutions was determined after hydrolytic equilibrium had been established. Freshly prepared stannous fluoride solutions hydrolyze within a short time with the formation of a white precipitate, probably composed of a mixture of $Sn(OH)F$ and $Sn(OH)_2$. Such solutions also attack glass, forming a film which flakes off after a time as iridescent scales. The quantity of precipitate formed during hydrolysis depends upon various factors, one of which in

this instance is the concentration of stannous fluoride. Such a solution in hydrolytic equilibrium produces more precipitate upon further dilution, and since part of the fluorine is retained by the precipitate in the form of Sn(OH)F, determination of the fluorine in the supernatant liquid was necessary before it was shaken with the enamel. The water used in making the stannous fluoride solutions was freed of oxygen by means of nitrogen which had been passed through a chromous chloride train. This oxygen-free nitrogen was bubbled through 5 L. of water at a rapid rate for 1 hour. All containers used for storing the water or stannous fluoride solutions were flushed with purified nitrogen before and after the addition of any liquid. A weighed quantity of stannous fluoride was dissolved in oxygen-free water to give a fluoride concentration of 100 μg per milliliter. Volumetric dilutions of this solution were made to give fluorine concentrations varying between 2.00 and 55.0 μg of fluoride per milliliter. These diluted solutions were transferred to 500 ml. glass-stoppered bottles which had been coated on the inside with paraffin. The solutions of stannous fluoride were stored under nitrogen and were not used for 30 days; this treatment ensured complete hydrolytic equilibrium. Analysis showed that 15 to 20 per cent of the fluorine was removed from solution by the precipitate formed during hydrolysis. When the solutions were shaken with enamel, the bottles used as containers were flushed with nitrogen after the solution had been added and then quickly sealed to exclude any atmospheric oxygen. In all other respects the procedure used for the stannous fluoride solutions did not differ from that used for the sodium fluoride solutions. Duplicate analyses were made on the stannous fluoride solutions before shaking with enamel and duplicate samples were shaken with enamel. The analysis of the original solution introduced an additional 3 per cent error in the fluorine analysis, but the error in F was about 10 per cent over the whole range of concentration because approximately 50 per cent of the total fluoride was removed from all of the stannous fluoride solutions by the enamel. In Table II are listed the data obtained from the analyses of the various solutions of stannous fluoride shaken with powdered enamel. These data are plotted in Fig. 1. It is evident that the removal of fluorine followed nearly a linear relationship when stannous fluoride was used. The slight curvature which is present shows an increasing slope as the concentration is increased, a behavior opposed to that of an adsorption isotherm. As in the case of sodium fluoride, later measurements with stannous fluoride showed that equilibrium had not been attained at the shaking time of 6 hours.

The pH of the sodium and stannous fluoride solutions were measured before shaking with the enamel. These data are listed in the respective tables. The pH of the stannous fluoride solutions decreased with increasing concentration of the salt, a fact which is to be expected with a salt of a very weak base and a moderately weak acid. The sodium fluoride solutions behaved similarly, although the pH change was not so great.

Rates of Removal of Fluorine From Solution by Dental Enamel.—Two series of solutions, stannous fluoride at 22.9 μg of fluorine per milliliter and sodium fluoride at 25.0 μg of fluorine per milliliter, were shaken with enamel

TABLE II

REMOVAL OF FLUORINE BY DENTAL ENAMEL FROM SOLUTIONS OF STANNOUS FLUORIDE

SAMPLE NUMBER	CONCENTRATION OF F IN SAMPLES		F REMOVED (ΔF)	pH OF ORIGINAL SOLUTION
	INITIAL (a) (μG/ML.)	FINAL (C) (μG/ML.)	(μG/GRAM ENAMEL)	
1	1.8_9	0.91	49	5.0
2	1.8_9	1.0_3	43	5.0
3	4.6_4	2.3_2	11_4	3.9
4	4.6_4	2.5_8	10_4	3.9
5	9.1_8	4.1_4	25_2	3.6
6	9.1_8	4.1_8	25_0	3.6
7	13.0_2	5.8_2	37_0	3.4_4
8	13.0_2	5.8_0	37_6	3.4_4
9	17.0_6	6.8_2	50_6	3.3_7
10	17.0_6	6.8_4	50_8	3.3_7
11	22.0_9	10.0_6	61_6	3.3_9
12	22.0_9	8.6_7	70_5	3.3_9
13	26.0_6	10.0_6	77_0	3.3_9
14	26.0_0	10.0_9	75_4	3.3_9
15	31.0_1	12.0_1	95_9	3.3_9
16	31.0_1	12.0_0	96_0	3.3_9
17	32.0_0	13.0_0	95_0	3.2_8
18	32.0_0	13.0_1	94_1	3.2_8
19	36.0_7	14.0_5	11_{10}	3.2_1
20	36.0_7	14.0_5	11_{10}	3.2_1
21	39.0_5	16.0_8	11_{00}	3.1_9
22	39.0_5	16.0_9	11_{30}	3.1_9
23	44.0_5	17.0_3	13_{05}	3.1_6
24		17.0_6	13_{50}	3.1_6

TABLE III

RATE OF REMOVAL OF FLUORIDE BY DENTAL ENAMEL FROM SOLUTIONS OF STANNOUS FLUORIDE, INITIAL CONCENTRATION OF FLUORIDE, 22.9 μG/ML.

SAMPLE NUMBER	C (μG/ML.)	TIME (MIN.)	$\frac{DC}{DT}$* IN μG/ML.$^{-1}$/MIN.$^{-1}$	FINAL pH
1	16.0_5	10	−0.118	6.2_0
2	13.0_{85}	39	−0.0696	6.5_0
3	12.0_{35}	60	−0.0450	6.5_0
4	11.0_{25}	100	−0.0196	6.6_0
5	8.0_{56}	540	-0.0030_7	6.5_6
6	8.0_{54}	728	-0.0016_6	6.7_0
7	7.2_2	1443	-0.0006_9	7.0_9

*Determined graphically.

TABLE IV

RATE OF REMOVAL OF FLUORIDE BY DENTAL ENAMEL FROM SOLUTIONS OF SODIUM FLUORIDE, ORIGINAL CONCENTRATION OF FLUORIDE, 25.0 μG/ML.

SAMPLE NUMBER	C (μG/ML.)	TIME (MIN.)	$\frac{DC}{DT}$* IN μG/ML.$^{-1}$/MIN.$^{-1}$	FINAL pH
1	24_1	11	-0.052_0	7.2_2
2	22_8	30	-0.013_1	7.4_1
3	23_2	60	-0.010_4	7.4_4
4	22_4	101	-0.0061_9	7.5_9
5	21_9	444	-0.0016_6	7.8_9
6	21_3	720	-0.0015_4	7.7_9
7	20_8	1440	-0.0047_7	7.6_5

*Determined graphically.

for various times up to 24 hours. The shaking and sampling procedures were the same as those described in Sections II and III. The data for these measurements are tabulated in Tables III and IV for the stannous fluoride and sodium fluoride solutions, respectively. Fig. 2 represents a plot of final fluoride concentration against time for both sodium fluoride and stannous fluoride.

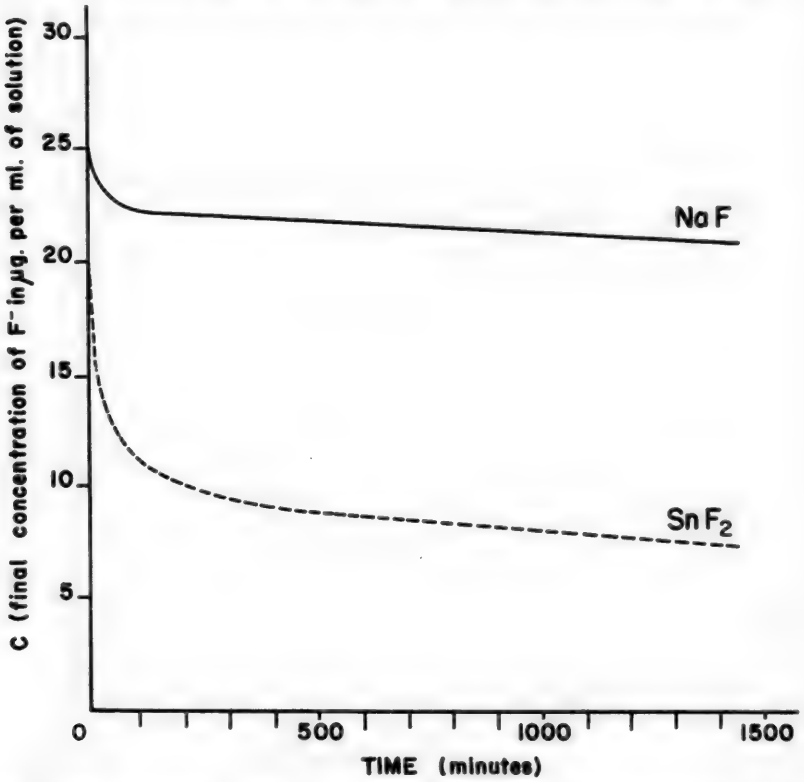

Fig. 2.—Rate of removal of fluorine by dental enamel from solutions of stannous fluoride and sodium fluoride.

DISCUSSION

The data in Tables I and II and the curves representing these data in Fig. 1 clearly showed that powdered enamel removes much greater quantities of fluorine from solutions of stannous fluoride than from those of sodium fluoride except at the very lowest concentrations. These more carefully conducted experiments corroborate those previously published[13] wherein it was shown that powdered enamel takes up larger amounts of fluorine from stannous fluoride solutions than from sodium fluoride regardless of the age of the solution. The greater removal of fluoride from stannous fluoride solutions may well be related to the lower pH levels of solutions of this salt, which hydrolyzes to give acidic solutions. Studies in this laboratory indicate that stannous fluoride is more effective in reducing the acid solubility of powdered enamel

when employed at low pH levels. Sodium fluoride and stannous fluoride were used in the present absorption studies at their natural hydrogen ion concentrations in order that the 2 salts might be compared at pH levels comparable to those prevailing in in vivo investigations.[12]

The data in Tables III and IV and the curves in Fig. 2 obtained in the investigation of the rate of removal of fluoride from solution again show that much more fluoride was removed from solutions of stannous fluoride than from those of sodium fluoride. It is to be regretted that the rate studies were inadvertently conducted after, rather than before, the adsorption studies. It is evident from the data that equilibrium had not been established after 6 hours of shaking of enamel with fluoride solutions in the absorption studies.

The role that increased concentrations of fluorine may have in increasing resistance to dental caries is not clear, since McClure[19] has demonstrated that there are no greater amounts of fluorine in caries-resistant teeth than in caries-susceptible teeth. However, it is inconceivable that fluorine is not associated in some manner with caries resistance, and it may well be that the amounts necessary to reduce dental caries appreciably are so small that analytical technics may not distinguish these differences. This is strengthened by the work of Myers, Hamilton, and Becks[20] using radioactive fluorine where they have shown that fluorine is taken up in measurable quantities only by the primary decalcified areas and not by normal enamel. Thus, the total amount may be diluted throughout the entire enamel area to such a degree that its detection may be missed.

SUMMARY

It has been shown that more fluorine is removed by powdered human dental enamel from aqueous solutions of stannous fluoride than from solutions of sodium fluoride containing stoichiometrically equivalent amounts of fluorine. This greater uptake from stannous fluoride solutions may be related to the greater hydrogen ion concentration of these solutions.

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EXPERIMENTAL VARIABLES AFFECTING CARIES PRODUCTION IN RATS FED DIETS CONTAINING PROCESSED FOODS

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TWO prior publications by the authors[1, 2] describe the production of caries in rats fed 2 relatively new diets neither of which contained customary cariogenic agents, i.e., coarse particles of corn or rice[3, 4] and excessive quantities of refined sugars.[5-7] These diets were responsible for the extensive production of smooth surface caries which appeared initially as opaque, elongated lesions in the enamel and which followed most generally the line of the gingival attachment. Occlusal caries was also present to some extent in rats fed the processed cereal diet but occurred to a limited extent or not at all in groups of rats fed diets containing skim milk powders. The smooth-surface caries particularly in its initial stages appeared strikingly similar to smooth-surface caries in human beings. Other reports have described similar smooth surface caries in hamsters[8] and rats.[9]

The results with the processed cereal diet (No. 586)[1] warranted the suggestion that heat-processed cereal foods were deserving of consideration for possible cariogenic properties. The results with diets containing the skim milk powders identified the cariogenicity of the skim milk powder with its commercial preparation, i.e., a spray process powder was less cariogenic than two roller process powders. Both powders had their cariogenic effect significantly increased by "dry" autoclaving for a period of 15 minutes. These results also suggested strongly that the heat processing required to prepare skim milk powders might be a factor causing cariogenicity of these particular diets.[2]

It is the purpose of this report to present additional data pertaining to caries production by these cereal and skim milk powder diets and to describe the results of several variations in the experimental regimen which affected the development of this particular type of rat caries. Data are presented also on the production of caries by a skim milk powder diet fed for different periods of time.

EXPERIMENTAL

As previously reported[1] the processed cereal diet (No. 586) had the following Composition: Cerelose (commercial glucose)—18.0 per cent; enriched rye bread (toasted)—25.0 per cent; enriched white bread (toasted)—25.0 per cent; cooked rolled oats (dry)—15.0 per cent; cooked yellow corn grits (dry)

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—15.0 per cent; and sodium chloride—2.0 per cent. A complete vitamin B concentrate was added to this mixture. The skim milk powder diets (No. 635 and others studied previously)[2] contained 35 per cent skim milk powder, 18 per cent cerelose, 45 per cent corn starch, and 2 per cent dehydrated liver. Diet 635, as studied herein, contained a roller process skim milk powder.[10] A vitamin A, D, and E concentrate was given weekly by mouth or in separate food cups as a supplement to both these diets. Other details regarding preparation of the diets may be found in the 2 previous articles.[1, 2]

The experimental rats were obtained from colonies maintained at the National Institutes of Health where the stock rat diet consists of Purina Lab Chow and a daily supplement of fresh greens. Both Sprague-Dawley and Holtzman strains were fed diet 635, and no strain differences were observed in the results. Only Holtzmans were fed diet 586. At weaning age all the rats were housed in screen-bottomed cages. Before weaning they were kept on a solid surface covered with sawdust. Food and distilled water were provided ad libitum. The diets were stored at approximately 5° C. With one exception (Experiment 3, Table I) all rats were placed on experiment at weaning age weighing 25 to 30 grams.

TABLE I

PRODUCTION OF DENTAL CARIES IN WHITE RATS FED PROCESSED CEREAL DIET 586 AND DIET 635 CONTAINING SKIM MILK POWDER

Experiment no.	1	2	3	4	5	6	7	8	9
Diet no.	586	586	586	635	635*	635	635	635	635
Rats per cage	1	2	3-10	1	3	2	9-12	6-12	8-11
Feeding regimen	A	B	C	A	B	B	B	B	B
No. of rats	38	60	123	29	29	39	30	45	45
Days on experiment	92	89	93	68	68	90	30	60	90
Average daily gain	0.71	0.51	0.27	2.06	2.00	1.92	2.62	1.93	1.91
Caries Incidence—Per Cent									
Carious rats	29.0	43.3	80.5	37.9	75.9	51.3	76.6	97.7	91.1
Occlusal carious rats	15.8	23.2	52.0	6.9	20.7	7.7	0.0	0.0	0.0
Surface carious rats	18.4	23.3	72.4	37.9	69.0	43.6	76.6	97.7	91.1
Caries Distribution and Severity (number per rat)									
Upper carious teeth	0.03	0.28	0.89	0.00	0.24	0.03	0.00	0.00	0.00
Lower carious teeth	0.63	1.30	2.00	0.83	2.21	1.00	2.10	2.76	2.87
Total carious teeth	0.66	1.58	2.89	0.83	2.45	1.03	2.10	2.76	2.87
Occlusal carious teeth	0.32	0.68	1.39	0.07	0.62	0.15	0.00	0.00	0.00
Surface carious teeth	0.34	0.98	2.98	0.76	1.97	0.87	2.10	2.76	2.87
Occlusal carious areas	0.37	1.25	1.55	0.10	0.93	0.21	0.00	0.00	0.00
Lingual carious areas	0.05	0.08	1.13	0.00	0.00	0.00	0.00	0.00	0.00
Buccal carious areas	0.10	1.42	4.34	1.00	3.17	1.15	2.70	4.82	5.02
Score per rat	0.87	4.35	8.41	1.28	5.28	2.72	2.76	5.58	5.84
Score per carious rat	3.01	10.03	10.45	3.36	6.95	5.30	3.61	5.71	6.41

*Contaminated diet.

The usual procedure in current studies with these diets is to house 2 rats per cage and to replenish the diet every 3 to 4 days without discarding uneaten food. This plan is referred to as feeding regimen B. In other studies fresh food was given daily and refused food discarded. This is designated as feeding regimen A. An effort was made to increase caries production on diet 586

by giving the diet to the mother rats when the young were born, and replenishing the diet every 3 to 4 days, i.e., feeding regimen C. With this arrangement the rats may start eating the diet at 15 to 18 days of age. Under this plan in Experiment 3, the litters were kept intact in 1 cage. Three to 10 littermates occupied 1 cage throughout the experiment.

In an additional study of what might occur as an extreme condition in a feeding regimen, diet 635 was treated as follows: Fresh diet was spread about 1½ inches deep beneath screen bottoms of cages housing 1 rat each per cage (Experiment 4, Table I). Diet 635 was thus heavily contaminated with excreta for several days, after which the solid excreta were removed, the diet dried, ground fine, and kept at room temperature before being fed to other rats (Experiment 5). Littermate rats were paired so that one-half received fresh diet 635 daily and were housed one rat per cage (Experiment 4). The other rats of the pairs received contaminated diet 635 according to regimen B and were housed 3 rats per cage (Experiment 5, Table I).

Another study (Experiments 7, 8, and 9, Table I) was planned to determine relative caries production after 30, 60, and 90 days' exposure to diet 635 under feeding regimen B. In an initial study, trios of littermate rats were housed at weaning age, 10 rats per cage. A succeeding study compared pair mates fed 60 and 90 days, respectively. Mortality varied the average number of rats per cage during the experiments, as shown in Table I. Rats were sacrificed at the end of 30, 60, and 90 days for caries diagnosis. At the termination of all feeding periods, the rats' heads were removed and autoclaved, and caries was diagnosed and scored as previously described.[1, 2]

In addition to new data shown in Table I, the results from a previous study with diet 635[2] are presented under Experiment 6 in the table. These rats were housed 2 per cage and were on experiment 90 days under feeding regimen B.

RESULTS

The data pertinent to these cariogenic diets are shown in Table I. The "pattern" of caries as produced by these diets is apparent in data showing (a) the per cent of carious rats in each group, (b) the distribution of occlusal vs. smooth surface caries, (c) the numbers of upper vs. lower carious teeth, (d) the carious involvement of lingual, buccal, and occlusal surfaces, (e) the average score based on all rats in the experimental group, and (f) the average score of the carious rats in the group. As previously noted, a particularly important effect of both of these diets is the extensive production of smooth surface caries. Diet 635 generally produced little or no fissure caries, i.e., involvement of occlusal surfaces. (See Experiments 7, 8, and 9.) Both diets produced caries in lower molar teeth more extensively than in upper molars and buccal surfaces were by far the most extensively and seriously affected.

The incidence and severity of caries are markedly different among the 3 experiments (1, 2, and 3) which were conducted under different feeding and housing regimens. The least successful regimen for caries production was individual housing and provision of fresh food daily (Experiment 1). A high

caries incidence (80.5 per cent) and an average score of 8.41 resulted when the diet was initiated before weaning, the food replenished at 3- to 4-day intervals, and the rats housed 3 to 10 per cage (Experiment 3).

Results for Experiment 2 are intermediate between the results for Experiments 1 and 3. Experiment 2 as compared with Experiment 1, however, indicates an advantage in caries production by housing 2 rats per cage and allowing the rats to consume food which had remained in the cage for as long as 3 to 4 days.

Data on diet 635, as fed under different conditions, are shown under Experiments 4, 5, and 6, Table I. The results for Experiment 9 also may be compared with Experiment 6. As was the case with diet 586 (Experiment 1), housing 1 rat per cage and providing fresh diet each day was the least successful arrangement for the production of caries. The per cent of rats with caries and the severity of caries were greatest in Experiment 5, where diet 635 had been contaminated with excreta as previously described, and feeding regimen B was followed for rats housed 3 per cage. An advantage in housing 2 rats per cage and replenishing the food at 3- to 4-day intervals (Experiment 6) rather than housing 1 rat per cage and giving fresh food daily is shown by comparison of the data for Experiments 4 and 6. This result with diet 635 is similar to that found for diet 586 (Experiment 1 vs. 2). A marked increase in both incidence and severity of smooth surface caries is recorded for Experiment 9 (8 to 11 rats per cage) vs. Experiment 6 (2 rats per cage).

Data for Experiments 7, 8, and 9, Table I, provide information on the relation of duration of exposure to diet 635, to caries development. It was somewhat surprising that so many rats, i.e., 76.6 per cent, developed caries with an average score of 2.76 by the end of 30 days on diet 635. After 60 days (Experiment 8), 97.7 per cent of the rats were carious with an average score of 5.58. This incidence and severity were not increased by an additional 30 days' exposure (Experiment 9).

DISCUSSION

There are available in this report actual caries data pertaining to diet 586 not previously published as well as additional data relative to diet 635. The results call attention to the fact that the caries response with these diets was influenced by the feeding and housing regimen. There has been no attempt, however, in these present studies to determine why the different feeding regimens produced different results. It is not uncommon in studies of experimental rat caries to fail to produce caries, or the results may be disconcertingly variable.[7] Inconsistent results have been attributed to inherent differences in caries susceptibility among different strains of rats, to management and feeding of the stock colony, or to subtle differences in the quality and composition of diet components.[6, 7] There is no doubt that these are factors of great importance in caries production. But it is suggested in the

light of these current studies that the experimental conditions which directly affect the rat and its diet also may have significant relations to experimental rat caries.

The promotion of caries by multiple housing is strongly suggested by comparison of the results for Experiments 6 and 9. Many more lesions and more severe caries resulted in Experiment 9 (8 to 11 rats per cage) vs. Experiment 6 (2 rats per cage). One other report[11] has presented data which also suggest that a greater incidence and severity of occlusal fissure caries resulted by housing 10 rather than 2 rats per cage.

The deterioration of protein in the skim milk powder of diet 635 is a possibility which must be considered if this diet remains several days in the rat's cage before being consumed.[12-14] In addition to the possibility of chemical changes occurring under such a feeding plan, there is every reason to expect an extensive microbial contamination not likely to occur when the diet is fed fresh every day. It is significant that the feeding conditions which provided for a minimum of microbial contamination and chemical deterioration (Experiments 1 and 4) resulted in the lowest incidence of caries. When optimum conditions for such changes were provided, as in Experiments 4, 5, and 9, caries production was most extensive and severe. Referring to Experiment 5, it is of interest to note that while this diet may or may not have been rendered more cariogenic by contamination by excreta, there was no apparent change in its nutritive value for growth. The rats in Experiment 4 (fresh diet daily) vs. Experiment 5 (contaminated diet) had the same daily weight gain.

In Experiment 3, diet 586 was available for consumption by the young rats prior to weaning. While it is not possible to attribute the high caries rates produced in this experiment to this factor alone, it may be noted that other cariogenic diets have been shown to be more effective if introduced prior to weaning.[7, 15] One explanation seems to be that susceptibility to caries is especially high in the early life of the rat. This idea is substantiated somewhat by the observation in Experiments 7, 8, and 9 that approximately two-thirds of the rats which eventually developed caries did so by the end of the first 30 days of exposure to the diet and developed no additional caries after 60 days.

SUMMARY

In support of previous evidence, data are presented demonstrating the production of dental caries in white rats by feeding a complex diet containing a combination of 4 processed cereal foods and also by feeding a diet containing a roller-processed skim milk powder. Heat-processed cereal foods and skim milk powders are being studied as the cariogenic agents in the diets. Variable factors of the experimental planning, including number of rats per cage and the feeding regimen, affected the caries outcome. Caries developed extensively during the first 30 days following weaning but seemed not to progress

after 60 days. An especially interesting type of smooth surface caries occurring most extensively on lower buccal areas was consistently developed in rats fed both these diets.

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THE CARIOGENICITY OF VARIOUS CORN DIETS IN THE RAT

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INTRODUCTION

THERE is little information available concerning the effect of different corn particle sizes with and without the addition of carbohydrate for the production of caries in the rat. Thus, the purpose of this paper is to show the effect of different corn diets, varying in the particle size of the corn as well as in the content of sugar, and to compare the effect of these diets in relation to initiation and progression of experimental dental caries in the rat.

EXPERIMENTAL

Approximately 250 weanling rats* were divided according to weight and sex into six experimental groups. All animals received the same moderately low-fluorine diet[1] and drinking water from the time they first began eating and drinking until they were 30 days old, at which time they were placed on the different experimental cariogenic diets. All the animals were housed in an air-conditioned room in metal cages with raised screen bottoms. The duration of the experiment was 140 days, and during this time the diets and drinking water were available to all the animals ad libitum. At the end of the experiment the animals were sacrificed by ether inhalation and the heads were prepared for caries examination as previously described.[1] The method of caries evaluation has also been reported.[2]

DATA AND DISCUSSION

The compositions of the different experimental diets are seen in Table I. The particle size distribution of the corn in these various diets is seen in Table II. The various diets are qualitatively similar, with the exception of M4, which is essentially a high-sugar diet. This diet was used because of reports[3, 4] that the incorporation of ethylene diaminetetraacetic acid produces a caries picture in rats more comparable to that in human beings and hamsters, especially in relation to smooth surface caries. This compound was fed at a 0.2 per cent level in the diet, which was used by other workers.[3] Diets M2, M3, and M6 differed only in the type of corn used in the diet: M2 had corn grits which were of a uniform particle size† and were not contaminated by pericarp or other extraneous material; M3 was composed of corn meal‡; M6 was composed of rather coarse corn[2] ground in these laboratories from whole yellow corn. The

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*Sprague Dawley Strain, furnished by Carl Wilson, Beech Grove, Ind.

†Supplied by the Evans Milling Co., Indianapolis, Indiana.

‡Degerminated yellow corn meal.

TABLE I. COMPOSITION OF THE DIFFERENT EXPERIMENTAL CARIOGENIC DIETS

DIETARY COMPONENTS	EXPERIMENTAL GROUPS					
	M1	M2	M3	M4	M5	M6
	(*Grams per six kilograms*)					
Whole milk powder	1,710	1,800	1,800		1,700	1,800
Yellow corn (I.U. ground)					2,760	3,840
Yellow corn (grits)	2,760	3,840				
Corn meal			3,840			
Granulated sugar (sucrose)	1,170			4,200	1,170	
Alfalfa	288	288	288		288	288
Irradiated yeast	12	12	12		12	12
Sodium chloride, iodized	60	60	60		60	60
Vitamin test casein				1,200		
Wesson oil				300		
Vitamin mixture				60		
Inorganic salts				228		
Ethylene diaminetetraacetic acid				12		
Percomorph oil				120 drops		

latter was sifted after grinding in such a manner as to make it comparable in physical composition to the corn grits, the essential difference between M2 and M6 being in the particle size of the corn. Diets M1 and M5 are also similar except for the nature of the corn, M1 containing corn grits and M5 corn ground in

TABLE II. PARTICLE SIZE DISTRIBUTION OF CORN COMPONENTS USED IN THE VARIOUS EXPERIMENTAL DIETS

CORN TYPE	PER CENT CORN RETAINED ON SPECIFIED SCREEN			
	8	10	20	PASS 20
I. U. ground corn	1	35	37	27
Corn grits	0	0	99	1
Corn meal	0	0	0	100

these laboratories. A difference, then, between the grits and the ground corn was that of particle size. Thus, if the chemical nature of the diets was of no consequence on caries production in the rat, the animals receiving M2, M3, and M6 and those receiving M1 and M5 should have similar caries scores. However, the findings in this experiment (Table III) indicate that the composition

TABLE III. THE EFFECT ON DENTAL CARIES IN THE RAT OF FEEDING VARIOUS CARIOGENIC DIETS FOR 140 DAYS

DIET NUMBER	SEX	NUMBER OF ANIMALS	NUMBER OF LESIONS	EXTENT†	S.I.P.‡
M1	Male	19	9.9 ± 1.4*	2.12	21.00
	Female	20	10.0 ± 0.9	2.52	25.20
M2	Male	18	7.9 ± 1.2	1.81	14.30
	Female	20	8.1 ± 0.8	1.79	14.50
M3	Male	21	7.0 ± 1.0	1.66	11.62
	Female	20	6.7 ± 0.6	1.40	9.42
M4	Male		All animals died		
	Female				
M5	Male	21	8.0 ± 1.1	1.61	12.84
	Female	20	8.1 ± 1.0	1.94	15.70
M6	Male	19	7.4 ± 0.9	1.69	12.50
	Female	20	7.1 ± 0.8	1.41	10.02

* Standard deviation.

†Calculated by determining the sum of all the individual severity numbers in a group, and calculating the mean.

‡Average severity per animal. Obtained by multiplying the number of lesions by the extent.

of the diet in relation to the nature of the corn or the sucrose content is of major importance in the cariogenicity of the diet. The greatest number of lesions, as well as the greatest severity, was found in those animals on diet M1, the diet composed of corn grits and sucrose. The SIP for these animals was almost twice that for any other group. One would expect that if the carbohydrate alone were the determining factor in rat caries, diet M5 would produce a similar number of lesions with similar extent values because it contains the same dietary components. Since this is not the case, one must conclude that the only other variable, namely, the corn, must be of significance in the caries-initiating process. Other work in this laboratory has indicated that a diet containing 65 per cent sucrose, but no corn, produces a very low caries incidence in the rat. Further, the compositions of M2, M3, and M6 differed only with regard to the type of corn used, yet the cariogenic effects of these diets were not alike. These findings indicate a possible superiority for caries production in those animals receiving experimental diet M2, which contained the corn grits. By comparing these findings with the data from M1, it is seen that the effect of the corn grits was really one of increasing caries experience in the rat. The caries findings for M3 and M6 were very similar.

The animals receiving the ethylene diaminetetraacetic acid all died before they were 100 days old. It is not known what caused this pronounced mortality, since other workers using essentially this same diet had good results when feeding this compound at the same level used in this experiment[3] and at a level 2.5 times as great.[4]

The effects on caries in those animals receiving diets M1 and M2 are of interest in that diet M2 contained 64 per cent corn grits while M1 contained 46 per cent, yet the caries score was considerably higher in the M1 animals. The fact that diet M1 contained sucrose while M2 did not must be of some significance in explaining the greater incidence of caries in these animals.

It has been a constant finding in this laboratory, as in others,[6] that carious lesions do not develop in the maxillary or mandibular third molars. However, in these studies it was not uncommon to find severe caries in the mandibular third molars in those animals receiving diets M1 and M5. In addition, a greater number of maxillary molars were found carious than in previous studies in which sucrose had not been used. Therefore, the presence of sucrose in the diets must contribute in some degree to the initiation or extension of caries in the rat, even though the same amount of sucrose in the two different diets did not produce the same degree of caries. The character of the carious lesion in those animals on the sucrose-containing diets was also considerably different from those on the diets containing no added sugar. It was the examiners' impression that the carious lesions appeared to be initiated throughout a wider area, as might take place throughout the entire area of a fissure, in contrast to the more typical corn-induced localized apex fissure caries. Thus, wider cavities appeared frequently and gave the impression of a more severe type of lesion. It was common also to note an intense brown pigmentation in these areas, the nature or significance of which is not known.

SUMMARY

The effects of different experimental diets were studied with regard to their ability to induce caries in the rat. The most effective diet for inducing and extending the carious lesion was one which contained 46 per cent corn grits and 19.5 per cent sucrose. A similar diet, except for the use of coarsely ground corn in place of corn grits and sucrose, reduced the average caries severity approximately 50 per cent. Three diets with identical constituents, but containing different types of corn, produced different caries pictures; the one with the largest and smallest particles produced about the same degree of caries, while corn grits, intermediate in size between the two, was more cariogenic. Increasing the amount of corn grits from 46 per cent to 64 per cent at the expense of sucrose did not increase caries. A greater number of lesions than usual were noted in the mandibular third molars, and an increase in the number of cavities was observed in the maxillary teeth in the animals on the sugar-containing diets. The lesions observed in the animals receiving the sucrose-containing diets were also different in size and character than those in the animals on a non-carbohydrate-containing diet.

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THE SYNTHESIS OF MUCINOUS POLYSACCHARIDES FROM SUCROSE BY ORAL BACTERIA

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THE synthesis of mucinous polysaccharides from sucrose by organisms of soil and plant origin to form slimy gels in fluid medium or mucinous growths on solid media containing this carbohydrate was recorded early in the annals of bacteriology.[1] The recognition of a similar synthesis from sucrose by bacteria derived from the oral and nasopharyngeal flora is more recent. Ørskov[2] and Ørskov and Paulsen[3] seem to have been the first to make this observation. Sherman, Niven, and Smiley[4] studied in detail the cultural factors influencing this polysaccharide formation by streptococci in their collection. They confirmed the original observation that the polysaccharide was a fructoside formed from sucrose and raffinose. The synthesis of the polyfructoside and fermentation of raffinose and inulin permitted a reasonable identification of these strains of streptococci as *Streptococcus salivarius*.

The immunogenic properties of the polysaccharides synthesized from sucrose by the nasopharyngeal bacteria have been reported by Neill, Hehre, and associates,[5-12] who pointed out the high degree of serologic specificity. In addition they determined the organisms responsible, the nature of the polysaccharides, and the enzymatic pathways by which this was accomplished. For details the reader is referred to these papers; the accomplishments may be summarized: Sucrose with its glucose and fructose units was the initial substrate for a sequence of enzyme reactions demonstrable with cell-free extracts which yielded levans from the fructoside unit and dextrans and amylopectins from the glucoside radical. Dextrans or levans alone or in combination were synthesized by certain nonhemolytic streptococci such as *Str. salivarius* (levan) and *Streptococcus s.b.e.* (dextran), whereas amylaceous polysaccharides were formed by gram-negative diplococci of the *Neisseria perflava* group, and *Corynebacterium diphtheriae*. Of special biochemical interest was the fact that the dextran was synthesized without the mediation of glucose 1-phosphate. Detection of levans and dextrans was greatly facilitated by serologic activity which permitted ready determination in high dilution by the precipitation technique. Of equal interest immunologically was the fact that either the levans or dextrans, irrespective of bacterial origin, were precipitated by antisera prepared against the respective polymer and showed little cross reactions with their optical opposites; that is, they were highly specific homogeneous

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groups in this respect. For example, the dextran synthesized by *Str. s.b.e.* was precipitated by antisera prepared against Types II, XII, and XX pneumococci whose capsular polysaccharides are dextrans. In contrast, the amylopectins (glycogen-starch) were readily detected by biochemical means because their iodophilic behavior gave the characteristic blue-black reaction with iodine.

Polysaccharide synthesis by oral bacteria seems to have been studied only in respect to *Str. salivarius* and primarily as a characteristic by which the species could be classified.[13] However, the Beldings[14-16] and Shiere, Georgi, and Ireland[17] have suggested the association of this species with the carious process. Our particular interest was in the possible relationship of these mucinous polysaccharides to the so-called mucinous plaque.* The present paper deals with the types and incidence of oral bacteria with this capacity, the polysaccharides produced, and some of the factors influencing their formation.

MATERIALS AND METHODS

Cultures.—A stock strain, S20B, of *Str. salivarius*† was used as the reference strain for the levan study. All other strains were obtained as subcultures from smooth and rough mucinous colonies of streptococci appearing on sucrose agar plates or colonies of gram-negative diplococci on blood agar plates, respectively, inoculated with specimens of saliva.

Medium.—The basic medium for the demonstration of mucinous polysaccharides was that described by Sherman, Niven, and Smiley.[13] The addition of calcium carbonate or gelatin was not tried. Hehre, Genghoff, and Neill[7] reported that the sugar should be carefully analyzed for presence of serologically reactive polysaccharides and that beet sugar was preferable to cane for satisfactory immunogenic study of these synthesized polymers. The limits of variation of this medium was tested by the stock strain (S20B) of *Str. salivarius.* It was found that the characteristic surface growth was formed over a rather wide range of the ingredients: smooth, soft, dome-shaped colonies occurred in concentrations of 1 to 3 per cent tryptose, 0 to 2 per cent dibasic potassium phosphate, 3 to 7 per cent sucrose, and a pH range of 6.0 to 8.0. Since the original formula was essentially optimal, it was used as recommended with the sucrose of beet origin.

Polysaccharides.—Levan: The isolation of levan for study in respect to optical properties was accomplished by the methods of Niven, Smiley, and Sherman[18] and Hehre, Genghoff, and Neill,[7] which were modified slightly for our needs. The quantitative study of amount and rate of levan formation was followed by a quantitative polyfructoside test based on the procedure of Hehre and Neill,[19] but adapted for "micro" volumes. The procedure for isolation of the levan was as follows:

> The levan of the supernatant of 1 L. of 5 per cent sucrose broth culture of *Str. salivarius* (S20B) was precipitated in the presence of sodium acetate (10 per

*Since this paper was prepared, a description of the various types of streptococcal colonies has been published by E. O. Morris, *Brit. D. J.* **96**: 97, 1954.

†Culture kindly supplied by Dr. J. M. Sherman, Cornell College, Ithaca, N. Y.

cent w/v) by 2.5 volumes ethyl alcohol (95 per cent) and overnight refrigeration. This precipitate was redissolved in water and reprecipitated. This precipitate was then dissolved and dialyzed against running water overnight. After filtering, the levan was precipitated once as previously described and once in the absence of sodium acetate. The final product was partially dried overnight at 55° C. and then with occasional stirring and grinding to maximum dryness at 100° to 110° C. Purity of product was tested quantitatively by the Shaffer, Hartman-Somogyi method for reducing sugars. Optical activity of the end product was determined in a Rudolf polarimeter.

The quantitative polyfructoside test as finally adopted was carried out as follows:

From 2 to 5 ml. of the supernatants of sucrose broth cultures of streptococci were put in 15 ml. centrifuge tubes. To these volumes, 2 drops of 1 per cent $CaCl_2$ and 2.5 to 3.0 volumes of 95 per cent ethyl alcohol were added. After maximum precipitation, the tubes were centrifuged and the supernatants discarded. The precipitates were dissolved in water and the procedure repeated. It was usually necessary to use warm water or to heat these suspensions slightly to dissolve the precipitate. Thin glass stirring rods were an aid. The supernatants were discarded and the precipitates dissolved in 3.0 ml. of 0.8N HCl, respectively, following which the tubes were covered and heated 15 minutes at 60° C. These acidic solutions were neutralized and transferred quantitatively to 25 ml. volumetric flasks and made up to volume with distilled water. Aliquots were then used for reducing sugar and fructose determinations.

Dextran: No attempt was made to isolate this polysaccharide because of the relative rarity of its occurrence.

Amylopectin: The iodophilic property of the amylopectin formed by the gram-negative diplococci allowed ready qualitative identification by its characteristic purplish black reaction with iodine. Hence, colonies synthesizing this polysaccharide could easily be detected by spraying the plates with Gram's iodine solution.

RESULTS

Incidence of Mucinous Colonies.—The numbers of organisms producing mucinous colonies on the basic medium were determined by dilution of specimens of saliva. The specimens were placed in a Kahn shaking machine for 3 to 5 minutes. Decimal dilutions were made in 9.0 ml. sterile water blanks to give 10^{-4}, 10^{-5}, and 10^{-6} concentrations of the original. From these, 0.1 ml. portions were placed on the surfaces of the test medium in duplicate and spread by means of sterile bent glass rods. Thus, the dilutions were 10^{-5}, 10^{-6}, and 10^{-7} for counting purposes. Mucinous colonies were always present in large numbers which were of the order of 5.0×10^8. This figure is at least one exponent higher than those normally attributed to oral bacterial counts. It may be partially due to the difficulty of obtaining evenly dispersed bacteria in the saliva for subsequent dilution with a single pipette. Likewise, the tendency of the colonies to fuse into streaks and masses made any counts subject to tremendous errors. It is doubtful if these numbers have much significance except to indicate the preponderance of this organism on this medium. Since these mucoid colonies were composed of masses of streptococci

in long chains and the polysaccharides formed by randomly selected strains, levan in nature, it was assumed on the basis of Sherman's work that these were representatives of a single species, *Str. salivarius*. No other bacterial type was observed to form similar mucoid colonies on this medium under either anaerobic or aerobic conditions. Aside from this quantitative aspect, the most interesting observation, which does not appear to have been previously described, was the marked variation in the appearance of these mucoid colonies. The stock strain (S20B) and the first specimens of saliva yielded nothing but soft, smooth, easily emulsified colonies 3 to 5 mm. in diameter and 2 to 3 mm. high (Fig. 1). Subsequent culture of specimens of saliva showed colonies of similar size but entirely different in appearance. Under low magnification with a binocular stereoscopic microscope (×7), these colonies looked like miniature peaks with sharply defined spurs and sides (Fig. 2). They were hard, difficult to emulsify, leathery to the touch, and comprised of streptococci. The polysaccharide formed by randomly selected strains was also levan in nature. As more specimens of saliva were cultured, and particularly if the plates were held for a few days, transitional forms appeared. These forms emerged from previously smooth colonies (Fig. 3). Thus it was made clear that this phenomenon was another example of microbic variation under the cultural conditions imposed. To ascertain if one or the other form or both appeared with varying age of the individuals, specimens of saliva were collected from groups of 30 students from the third, fifth, seventh, ninth, and eleventh grades as well as from two dental classes of 150 students. The mucinous colonies were always present in numbers already noted; there did seem to be a predominance of the smooth type in the younger age groups and of the rough in the older (dental students). The significance, if any, of this transition was not apparent. There was no correlation between numbers and type of these mucinous-producing streptococci and the lactobacillus counts of the respective specimens of saliva.

Polysaccharides Formed.—Levan: After standardization of the method for isolation and identification of the polysaccharide (levan) formed by the stock strain of *Str. salivarius* was accomplished, this was duplicated for several strains of smooth, mucinous strains of streptococci cultured from the stock medium and similar to those formed by the S20B strain. On the other hand, while the R colonies were undoubtedly variants of the S form and should have the same optically active polysaccharide, it was felt necessary to test this point. For this study, 55 strains, about equally divided between the S and R types, were obtained from 55 specimens of saliva randomly selected from the cultures on children and dental students. These were cultured in 5 per cent sucrose broth in tubes and the supernatant tested at 48 hours by the quantitative polyfructoside test. All but 2 of these strains produced only levan. The remaining 2 strains formed mixtures of levan and probably dextran, although this component was not identified. These results would merely confirm the expectancy that levan was the chief mucinous polysaccharide synthesized from sucrose by oral streptococci irrespective of colony type.

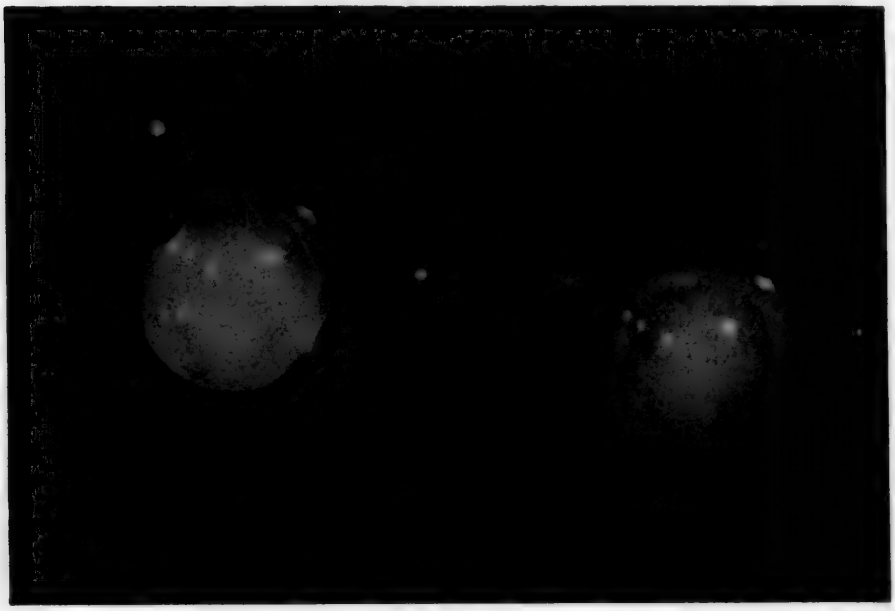

Fig. 1.—Smooth mucinous colonies formed on 5 per cent sucrose agar by *Str. salivarius* (S20B) and other oral strains of streptococci (×10, reduced ¼).

Fig. 2.—Rough mucinous colonies formed on 5 per cent sucrose agar by oral strains of streptococci (×10, reduced ¼).

The quantitative aspect of levan formation was also of interest, and in the study of its formation it was clearly shown that the amount and rate of formation could be profoundly altered by pH and number of organisms in the inocula. Thus, 1 L. of 5 per cent sucrose broth inoculated with 0.5 ml. of an 18-hour meat mash culture of *Str. salivarius* (S20B) and incubated 5 days at 37° C. yielded only 1 Gm. of levan at the end of the incubation period. As might be expected, little of the sucrose was hydrolyzed as shown by periodic removal of portions of the culture, centrifuging, and testing the supernatant quantitatively for reducing sugars as an index of levan formation. Neither changing the components of the medium nor changing the time and temperature of incubation increased this yield. The answer for the most part was in the pH of the culture which

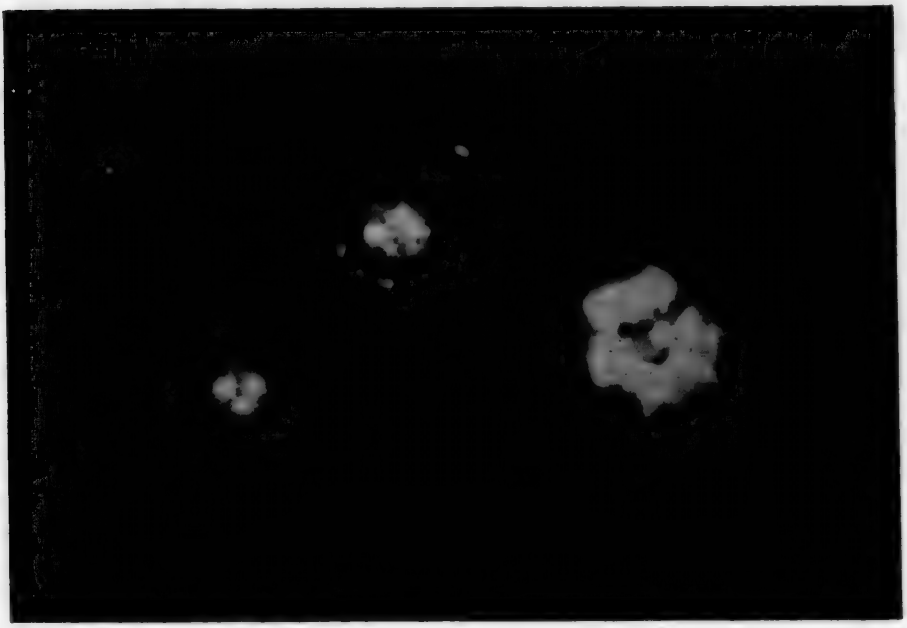

Fig. 3.—Intermediate colony types of mucinous colonies formed on 5 per cent sucrose by oral strains of streptococci (×10, reduced ¼).

in 16 to 24 hours dropped from an initial pH 7.2 to pH 3.5. This latter figure was far below the optimal value, pH 5.5, reported by Hestrin and Avineri-Shapiro[20] for the enzyme responsible for this synthesis. Daily or twice daily neutralization with sterile 2.5N NaOH then allowed the enzyme to continue transforming the fructose moiety into the polymer. For example, when 1 L. of 5 per cent sucrose broth was inoculated with 5.0 ml. of an eighteen-hour glucose broth culture of the S20B strain of *Str. salivarius,* and neutralized at 8 and 24 hours, 13 Gm. of levan was obtained after 30 hours when a maximum of reducing sugar was present in the broth culture incubated at 37° C. The exact quantitative relationship between reducing sugars and amount of levan

formed was not too clear under the conditions imposed in a living culture. Cell-free extracts were not used. However, a maximal yield of levan (13 Gm.) was obtained when one-half the sucrose could be accounted for as reducing sugar. The effect of neutralization on the reducing sugar content of sucrose broth cultures is illustrated in Fig. 4.

The formation of levan could also be further accelerated by increasing the ratio of bacterial cells to the substrate, as Stephan and Hemmens[21] did in their experiments dealing with pH changes in mass cultures of various oral bacteria either in pure culture or in combinations. Thus, with bacterial creams of washed cells harvested from glucose agar surfaces and made up to 33 per cent volumes in 5 per cent sucrose broth, reducing sugars and levan formation appeared in 5 minutes and reached a maximum in 20 minutes. The results of such an experiment are shown in Fig. 5.

Dextran: The presence of dextran-forming streptococci in the nasopharyngeal flora, while qualitatively common, is most difficult to determine. Thus, while Hehre[10] could isolate these streptococci from the throats of 9 of 18 healthy young adults, he did so by randomly subculturing 50 to 70 colonies from each of the blood agar plate cultures. Of the total of 1,076 isolates, only 28 strains produced dextran in the supernatants of sucrose broth cultures as shown by the precipitation technics using Type II pneumococcus antiserum as the antibody. This represented less than 3 per cent of all the colonies. It is obvious that many individuals could be tested without success on the basis of single subcultures, or 2 or 3 subcultures at the most. It would probably explain our failure to obtain a dextran-forming streptococcus in several hundred strains of nonhemolytic streptococci from a relatively large group of subjects. These organisms were subcultured to sucrose broth and the supernatants tested after 48 hours with Types II, XII, and XX pneumococcus antisera.* In no case was precipitation observed. Therefore, it was assumed that dextran synthesis would not have any importance to our problem since its incidence was not likely to be any higher in the saliva than the nasopharynx.

Amylopectin: Hehre and Hamilton[11] also reported the production of amylopectins by some of the gram-negative diplococci of the nasopharyngeal flora. They were able to demonstrate their presence better by anaerobic than by aerobic methods. The presence of this polymer was then readily brought out by spraying the plates with Gram's iodine solution since it reacted with the polysaccharide to produce a characteristic purple color. In our study, determination of amylopectin formation was complicated by the overwhelming number of levan producers. In fact, in all the preceding series only one strain was recovered by dilution whose colonies blackened with iodine irrespective of anaerobic or aerobic incubation. The selective action of penicillin as suggested by Dr. Hehre was tried: Dilutions of saliva were cultured on plates of 5 per cent sucrose agar containing 10, 1.0, and 0.1 units of penicillin

*Antisera kindly furnished by Dr. H. J. Cox, of Lederle Laboratories, Pearl River, N. Y.

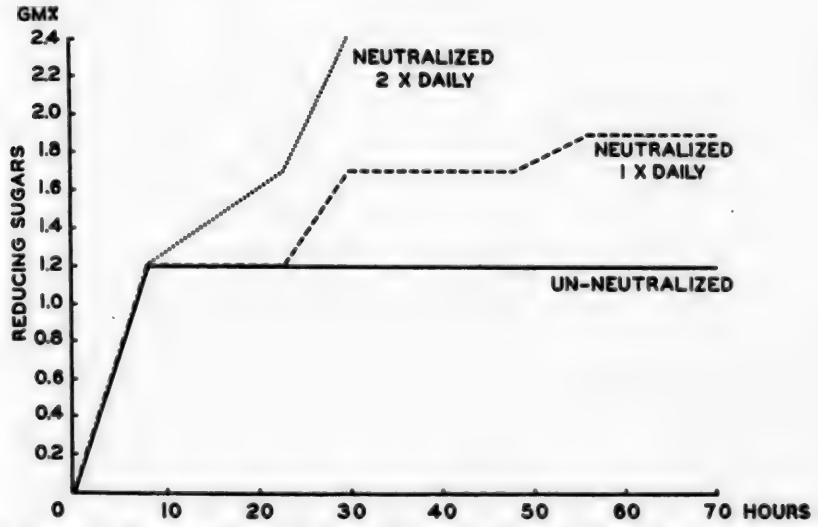

Fig. 4.—Effect of neutralization on formation of reducing sugars by *Str. salivarius* (S20B) in 5 per cent sucrose broth.

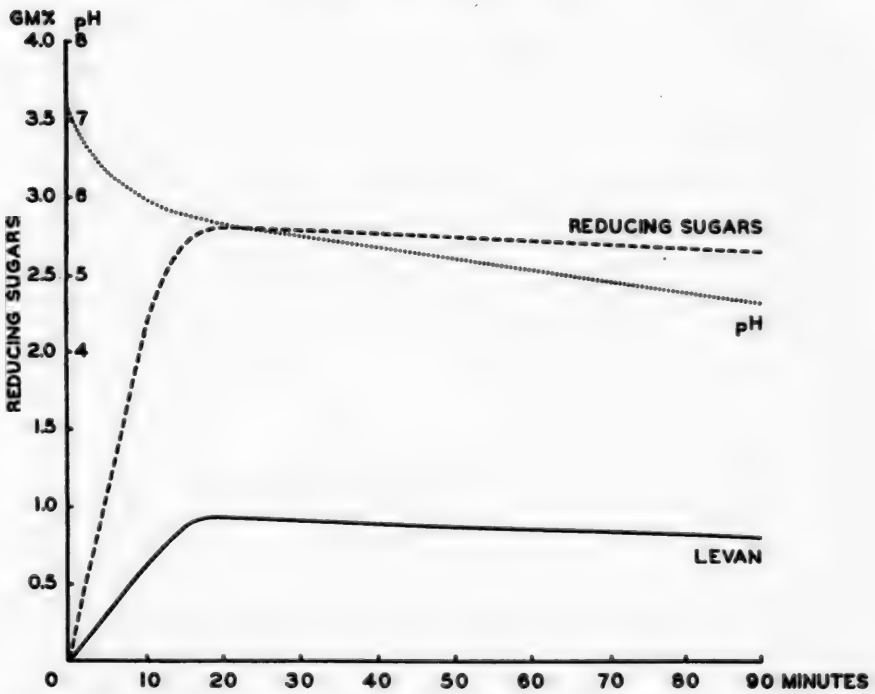

Fig. 5.—Effect of mass numbers (33 per cent cells per volume) of *Str. salivarius* (S20B) on formation of reducing sugars, levan, and pH in 5 per cent sucrose broth.

per milliliter medium. The oral flora proved to be highly sensitive to penicillin; scarcely any colonies appeared in the 1.0 unit per milliliter concentration, but there was no appreciable difference between aerobic and anaerobic plates containing 0.1 unit of this antibiotic. Resort was made, therefore, to streaking a loopful of the 1-10 dilution of saliva on sucrose agar and spraying the plate with Gram's iodine. This proved fairly satisfactory but results were frequently masked by overgrowth of the levan-forming colonies. Enough positive results were obtained to make us feel that as far as the oral

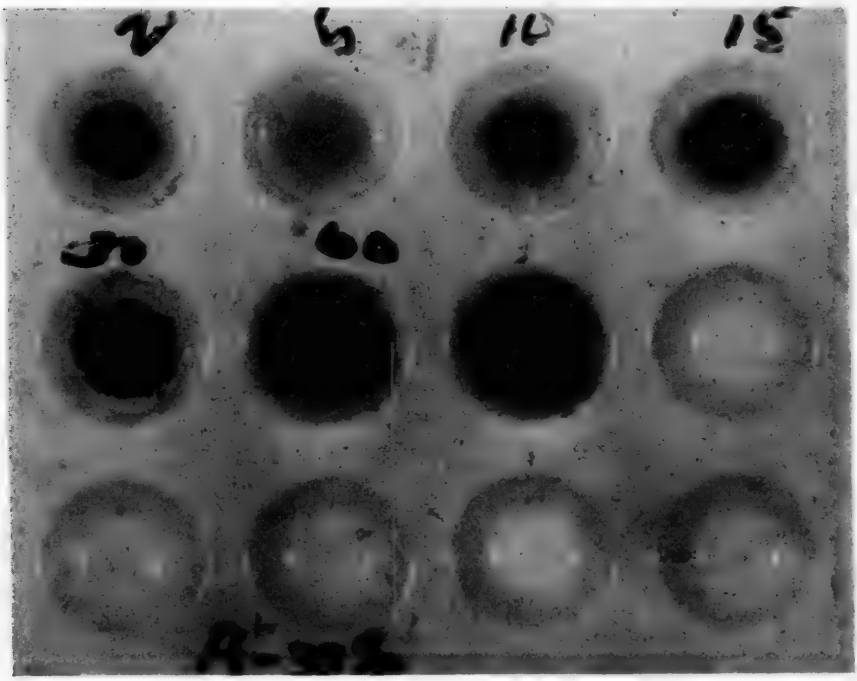

Fig. 6.—Effect of mass numbers (33 per cent per volume) of an oral Neisseria strain on formation of amylopectin in 5 per cent sucrose broth (minutes).

flora was concerned, amylopectin formation was limited to the gram-negative diplococci. A series of 100 specimens of saliva were cultured in this manner on blood agar, and representative colonies of gram-negative diplococci were present in more than 90 per cent of the specimens and of these at least 75 per cent were capable of synthesizing this polymer. The majority of these positive strains were of the yellow-pigmented types, whose cultural and serologic properties have recently been described by Pelczar, Faber, Hajek, and Warner,[22, 23] although in our study a few nonpigmented strains also had this property of amylopectin synthesis. There was a marked contrast between the amounts of polysaccharide formed; that is, the levans were produced in abundance as reflected by the size of the colonies on sucrose agar,

whereas the colonies of gram-negative diplococci did not appear differently on either blood or sucrose media. Anaerobic cultures failed to show any significant differences from the aerobic. This was in contrast to the findings of Langford, Faber, and Pelczar[24] and Douglas.[25] Our experience suggested that 5 per cent sucrose agar was far from an ideal medium for the primary isolation of the gram-negative diplococci.

Neither the precise nature of the amylopectin nor the amount was determined, but the rate of formation was studied in a manner similar to that for the levans. Bacterial creams of gram-negative diplococci of an oral strain known to form amylopectin were harvested from trypticase soy agar surfaces, washed twice in saline solution and made up to 10, 20, and 33 per cent volumes in sucrose broth, and placed in a water bath at 37° C. The amylopectin could be demonstrated in 30 to 60 minutes by simply pipetting a small amount of the suspension to a spot plate and adding iodine as shown in Fig. 6.

DISCUSSION

From the foregoing description it is clear that the formation of mucinous polysaccharides in vitro from sucrose is a capacity of only 2 or 3 of the bacterial types comprising the aerobic oral flora. One, the levan-forming *Str. salivarius,* occurs in astronomical numbers; another, the amylopectin-synthesizing gram-negative diplococcus is found quite constantly but in much smaller numbers than the streptococci; the third, dextran-forming streptococci, occurs so rarely as to have no apparent significance in the over-all process. The interesting thing about the synthesis of either levan or amylopectin is that it can be shown to take place within minutes under appropriate conditions; that is, mass bacteria in a small amount of substrate. Since this condition occurs on the tooth surface when bacterial cells in the plaque are repeatedly exposed to relatively small amounts of fluid substrate, it is reasonable to conclude that localized polysaccharide synthesis occurs naturally following ingestion of sucrose. This process would also be influenced by the prompt degradation of the glucose and part of the fructose to reduce the pH in a manner similar to that shown by Stephan[26] to occur on tooth surfaces after an oral glucose rinse.

The Beldings[14-16] indicated that *Str. salivarius* has some role in the caries process because they could be almost eliminated when the dietary carbohydrate was restricted. This change paralleled that normally accepted for decrease in numbers of lactobacilli following reduction of dietary carbohydrate. Although we made no effort to repeat this observation with clinic patients under nutritional study, we did not observe such changes under cultural conditions; that is, mucinous colonies were found in large numbers in all specimens of saliva irrespective of caries activity. This was particularly true of the dental students whose clinical status had been measured during their school attendance and confirmed in the majority of cases by lactobacillus counts. The constancy of *Str. salivarius* and the increasing amount of caries experience with age as encountered in average school populations could explain

the correlation coefficient (0.43) reported by Shiere, Georgi, and Ireland[17] for *Str. salivarius* counts and number of DMF teeth, but what the significance of this figure is in respect to the known rise and fall in caries activity of an individual would be most difficult to say.

SUMMARY

Of the complex oral aerobic flora only 2 types of bacteria were shown to be capable of synthesizing mucinous polysaccharides from sucrose; *Str. salivarius,* which formed levan, and gram-negative diplococci, which produced amylopectin. Greatly increased yields of levan were obtained by periodic neutralization of the sucrose broth cultures. Under conditions of mass bacteria and small amounts of substrate, synthesis of both levan and amylopectin was accomplished within a matter of minutes. Because of the water-soluble nature of the mucinous polysaccharides, their participation in the formation of dental mucinous placques was believed unlikely.

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METABOLIC STUDIES OF A TYPE OF LACTOBACILLUS ASSOCIATED WITH CARIES

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IN PREVIOUS studies of fermentative types of lactobacilli found in the saliva, it appeared that rhamnose fermenters designated as Type I were found more often than rhamnose nonfermenters in those individuals with extensive caries.[1, 2] Of the 2 types of rhamnose nonfermenters, one (Type II) was found in many more of the saliva specimens from both carious and noncarious individuals than the other. It seemed of interest to determine whether Type I had metabolic properties which would indicate that it would be more active than Type II in producing caries. Therefore, acid production of resting cells of both types was determined manometrically using glucose, sucrose, and maltose as substrates. Turbidities and acid production in glucose of growing cells were also compared. Since sucrose may be broken down to acid, and as little acid was produced in manometric determinations with resting cells, the ability to hydrolyze sucrose was determined. A comparison of the O_2 uptake of the 2 types was also made.

Finally, as one of us[3] had previously shown that lactobacilli grown in media containing NaF have decreased ability to produce acid, 5 strains of Type I were grown in NaF and then compared with parent strains for growth rate, acid-producing ability of growing cells, and acid-producing ability of resting cells in the absence of NaF. Such induced changes as might be observed under these artificial conditions might also occur with lactobacilli continually exposed to small amounts of fluoride in drinking water.

EXPERIMENTAL PROCEDURES AND RESULTS

Acid Production of Resting Cells From Dextrose.—Ten strains of rhamnose-fermenting (Type I) and 10 strains of rhamnose-nonfermenting lactobacilli (Type II) isolated from the saliva of children aged 11 to 14 years were used to compare the amount of acid produced from resting cells of each group. Organisms were harvested from tryptose phosphate broth (TPB) containing 0.2 per cent yeast extract, after 18, 24, and 36 hours' growth. Acid production was measured as microliters of CO_2 produced from bicarbonate in a Warburg respirometer. The solutions used in the flasks were essentially those of Perlman.[4] The manometers were flushed with 95 per cent N_2 and 5 per cent CO_2. The values in Table I are averages of 2 or 3 values determined simultaneously with a given organism. In the first experiment with organisms harvested at 18 hours, 7 of 10 Type I strains produced 200 or more microliters of CO_2 per

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TABLE I

ACID PRODUCED FROM GLUCOSE BY RESTING CELLS, RHAMNOSE-FERMENTING (TYPE I) AND NONFERMENTING (TYPE II) LACTOBACILLI

TOTAL $\mu l/CO_2/2$ HOURS

TIME HARVESTED	18 HOURS				24 HOURS		36 HOURS	
TYPE OF	EXP. A		EXP. B					
LACTOBACILLI	I	II	I	II	I	II	I	II
Strain 1	246	97	221	138	301	176	75	118
Strain 2	248	163	263	161	233	169	162	100
Strain 3	208	147	234	170	173	127	108	75
Strain 4	137	103	169	107	133	143	96	105
Strain 5	216	123	204	120	239	154		
Strain 6	188	62	--	78	155	88		
Strain 7	135	122	160	--	86	150		
Strain 8	253	163	178	157	232	121		
Strain 9	287	177	161	140	210	158		
Strain 10	226	136	488	160	175	108		
Median values	220	129	204	140	195	146		

Contents of flasks: 0.5 ml. bacteria suspended in H_2O, 0.2 ml. of 0.3 M $NaHCO_3$, 0.3 ml. of 0.1 M dextrose
0.8 ml. salt solution, and 1.4 ml. H_2O: Total vol. 3.2 ml. pH 7.4
Salt solution consisted of $MgSO_4$ .7 H_2O 4%
NaCl 0.2%
$MnSO_4$ H_2O 0.2%
Determination carried out in an atmosphere of 95% N_2 and 5% CO_2.

TABLE II

GROWTH AND ACID PRODUCTION IN GLUCOSE BROTH STRAINS OF TYPE I AND OF TYPE II LACTOBACILLI
(TIME OF GROWTH 36 HOURS)

STRAIN	ML. ACID PRODUCED PER 100 ML. MEDIUM		KLETT READING	
	TYPE I	TYPE II	TYPE I	TYPE II
1	26	22	280	220
2	26	22	260	218
3	26	20	255	212
4	25	20	250	212
5	25	20	240	210
6	24	20	236	208
7	24	20	229	208
8	24	18	220	204
9	22	16	220	196
10	22	16	220	190
11	22	16	218	190
12	21	14	212	182
13	20	14	204	177
14	19	14	200	175
15	18		198	
16	18		195	
17	18		192	
18	16		180	

milligram of dry bacteria in 2 hours. None of the Type II strains reached this value. Subsequent experiments in which the organisms were harvested at 18 hours and 24 hours showed the Type I group to produce more acid. At 36 hours the values were considerably lower for 4 strains of each type and showed no appreciable difference between the types. If harvested at the time of greatest activity, resting cells of Type I apparently produce more acid under anaerobic conditions than do Type II.

Acid Production From Dextrose and Turbidity Produced by Growing Cells. —One hundred milliliter portions of dextrose broth (Difco) were inoculated with 1 ml. of a 24-hour broth culture of 18 strains of Type I and 14 strains of Type II lactobacilli. After 12, 18, 24, and 36 hours, 1 ml. amounts were removed and titrated with 0.1 N NaOH. The maximum amount of acid was produced at 36 hours. Values are reported at 36 hours in Table II. At this same

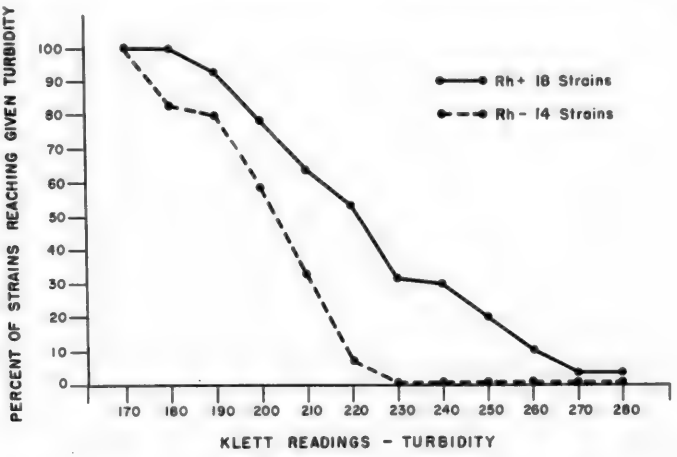

Fig. 1.—Turbidity reached in 36 hours by Type I and Type II strains of lactobacilli.

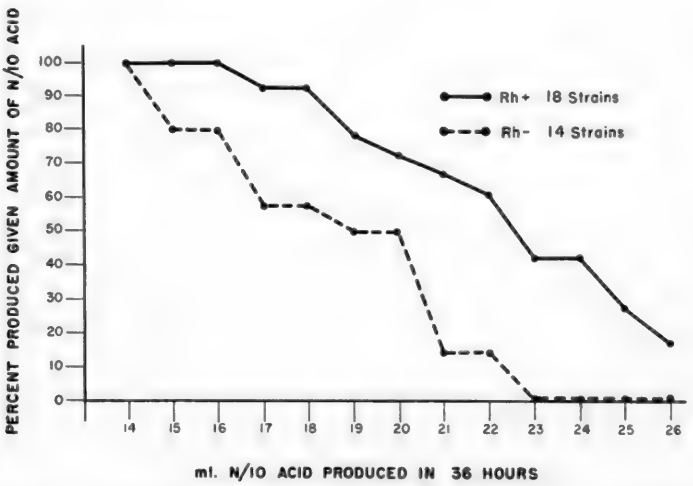

Fig. 2.—Acid produced in 36 hours by Type I and Type II lactobacilli.

time 5 ml. of broth was removed and turbidity measurements were made on the Klett-Summerson photoelectric colorimeter using a blue filter. A water blank read 100. Acid production and growth are compared graphically in Figs. 1

and 2. Fig. 1 shows that a larger percentage of Type I than of Type II produced high turbidities. A larger percentage of Type I also produced greater amounts of acid.

Metabolism of Maltose and Sucrose.—Maltose and sucrose were used as substrates in glycolytic experiments as dextrose was in the manometric determinations given previously. With several strains of each type of lactobacillus, values of 20 μl of CO_2 or less per milligram of dried bacteria were obtained after 4 hours' shaking in the flasks. Growing the organisms in sucrose and maltose broth did not change their ability to produce acid from these substrates. There was no appreciable difference in the small amount of CO_2 produced by the two different types.

Sucrose is hydrolyzed to dextrose by some bacteria. If these lactobacilli can hydrolyze sucrose to dextrose, then acid could be produced by the same enzyme systems shown to be present in our previous experiments. To determine whether these organisms were capable of hydrolyzing sucrose, 3 strains of each type were grown in TPB, and their ability to produce reducing sugars from sucrose determined. This was carried out by harvesting the organisms from the broth after 24 to 48 hours, washing twice in distilled water, and making up a heavy suspension in water. One milliliter of this suspension was inoculated into 25 ml. of 1 per cent sucrose in water and incubated at 37° C. for 24 and 48 hours. One milliliter amounts were removed from each flask, and reducing sugar was determined by Nelson's photometric method.[5] Table III shows these values for 2 separate determinations on the 6 strains. Only a small part of the sucrose was changed to reducing sugar by any of the lactobacilli, but larger amounts were produced by Type II than by Type I.

TABLE III
HYDROLYSIS OF SUCROSE BY TYPE I AND TYPE II LACTOBACILLI

		MG. RED SUGAR/100 ML. OF 1% SUCROSE			
		24 HOURS		48 HOURS	
TYPE	STRAIN	EXP. 1	EXP. 2	EXP. 1	EXP. 2
I	1	0.9	0.5	1.0	0.9
	2	1.3	--	1.8	1.8
	3	0.9	--	1.6	1.0
II	1	17.0	18.4	18.9	19.3
	2	25.8	30.7	39.2	31.3
	3	36.9	41.5	43.8	50.7

These 6 strains were also grown in sucrose broth preliminary to testing out their ability to utilize sucrose. Values for all were much lower than when the cells were harvested after having multiplied in dextrose broth. However, the values were again higher for the 3 strains of Type II than they were for the 3 strains of Type I.

Oxygen Uptake of Resting Cells.—The ability to take up O_2 with both glucose and sucrose was determined for 3 strains of Type I and 3 strains of Type II lactobacilli. The determinations were carried out with a Warburg respirometer at 37° C. using the methods described by Umbreit, Burris, and Stauffer.[6]

The values for all strains were low (less than 25 μl/mg./hr.), and there was no difference between the values found for Type I and those found for Type II.

Effect of Growing in the Presence of NaF on Growth and Acid Production in a NaF-Free Medium.—It has previously been shown that growing lactobacilli in media containing NaF lowered their ability to produce acid from glucose, even when no fluoride was present.[3] Because Type I lactobacilli appeared to be more often associated with caries than Type II, it was thought that the effect of NaF in drinking water in preventing caries might be due to a change of these organisms after long contact with the NaF. Five strains of Type I were transferred every 4 or 5 days from TPB containing 10 ppm NaF to broth containing increasing concentrations, until 100 ppm was reached. They were then transferred many times in broth containing this concentration of fluoride. Two strains were transferred 100 times and 3 strains 42 times. These organisms were then transferred to tubes of TPB, and after 24 hours' incubation inoculated into flasks of broth. They were harvested after 18 hours and the cells were used for inoculating dextrose broth for acid titration, for growth determinations by turbidity, and for glycolytic activity in the Warburg respirometer. As was previously shown, the acid produced by growing cells in dextrose broth was less with the fluoride-trained strains than with parent strains. The fluoride strains did not grow as rapidly. Table IV shows a series of comparative glycolytic values. Four of the five fluoride-grown strains show a decreased glycolytic activity.

TABLE IV
GLYCOLYTIC ACTIVITY OF TYPE I LACTOBACILLI BEFORE AND AFTER GROWTH IN NaF

STRAINS	μl CO_2/MG./2 HOURS	
	EXP. 1	EXP. 2
1 P	101.0	95.0
1 F 100*	36.8	28.6
2 P	143.9	180.0
2 F 100	75.0	104.0
3 P	111.3	
3 F 42	112.4	
4 P	179.5	
4 F 42	39.8	
5 P	251.5	180.5
5 F 42	107.0	116.6

P = Parent strain.
F = Fluoride-trained strain.
*Number of transfers in media containing F.

Kitchen, Robinson, Permar, and Imhoff[7] have shown that topical application of NaF does not change the lactobacillus count, and Rickles and Becks[8] have confirmed this and found that the acidogenic properties of the oral flora are unchanged. Lactobacillus counts of children drinking water containing fluoride have been generally lower[2, 9] than of those drinking fluoride-free water. Whether or not over a long period the type of lactobacillus found in the saliva will change upon contact with fluoride in the drinking water has not been proved, although it has been shown that after 1 year children who had been drinking water containing 1 ppm fluoride did not have a smaller percentage of Type I in the saliva.[10] Whether the Type I lactobacilli isolated had

decreased acidogenic properties, without otherwise changing type, was not determined. It still seems that one possible explanation for the mode of action of fluorine may be that prolonged contact with it may induce changes in the properties of the lactobacilli in the mouth. Other factors are, no doubt, in operation, too.

DISCUSSION

Since Type I lactobacilli have been found somewhat more often in the saliva of those individuals with extensive caries than have Type II, it is interesting to find that this type also has some properties that might make it more capable of producing caries. The ability to produce greater amounts of acid by most Type I strains, and to grow faster, would indicate this type to be more injurious if we accept the acidogenic theory of the cause of caries. The fact that the resting cells of Type I produce more acid than cells of Type II shows that the increased acid production is more than a reflection of the more rapid growth rate of the Type I strains. The reversal of activity of the 2 types in their sucrose-hydrolyzing properties is difficult to evaluate in relation to caries production, but does serve to emphasize that there are several essential differences in the types, which eventually may prove them to be separate species.

Although it had been shown that growing in the presence of NaF induces a loss of acidogenic properties, it was gratifying to be able to repeat this with these newly isolated strains and to show that the loss of acid-producing properties is not entirely due to a slower growth rate, but is found in resting cells of the trained strains as well.

SUMMARY

1. A study of the glycolytic activity of the resting cells of 10 strains of Type I and 10 strains of Type II lactobacilli showed the Type I strains to have greater glycolytic activity. The Type I strains have previously been shown to be more closely associated with caries than were Type II.

2. A study of the rate of growth and the acid production from dextrose by growing cells of 18 strains of Type I and 14 strains of Type II showed that a greater percentage of Type I strains produce larger amounts of acid and grow faster than Type II strains.

3. Very little acid was produced by resting cells of either type from sucrose or maltose.

4. Type II cells produce small but appreciable amounts of reducing sugar from sucrose in 24 hours. This is an additional property which is definitely different in the 2 types and further establishes them as 2 distinct types of lactobacillus.

5. O_2 uptake was very low for both types and no appreciable difference was seen.

6. Strains of Type I grown in media containing NaF produced less acid than the parent strains when grown in dextrose broth containing no fluoride. They also grew more slowly and the glycolytic activity of the resting cells was impaired.

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EVALUATION OF CARIES ACTIVITY BY THE BUFFERS OF SALIVA

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WITHIN the complex mechanism the organism utilizes for self-protection against dental caries there are a series of unknown factors which lead to a total absence of caries or to rampant caries. A number of theories have been put forth to explain such conditions and, although they have clarified many obscure points, it is evident that the total solution has not yet been reached. Because saliva is the normal environment of the tooth crown, it is believed that changes in it will be reflected in the physiology of the tooth. Therefore, we think it necessary to study all the facts presented by saliva. To accomplish our purpose we have chosen to investigate one of them, its buffer power, in an attempt to solve the problem of the etiology of dental caries.

Dreizen, Mann, Cline, and Spies[1] have demonstrated that the concentrations of saliva buffers vary in relation to the number of cavities. On the basis of this result they suggested a test to evaluate saliva activity. Fosdick[2] stated that carbohydrates are degraded in the mouth by the action of enzymes and lactobacillus. Stephan[3] demonstrated that the tooth is decalcified in a medium with a maximum pH of 5. These results are corroborated by Muracciole, Rey Millares, Martinez, and Rocco.[4]

From these findings we are of the opinion that when acids are formed in the mouth, the organism is compelled to react against them, and the most effective reaction is neutralization of the acids by the saliva buffers. It must be remembered that the organism protects itself against internal pH variations by blood buffers.

A subject with many cavities in his teeth lacks defense. We are not going to discuss whether this defense is general or local, but we believe that it need not be localized within the buccal cavity, but may have its basis in the whole organism. Fosdick[5] has suggested that the buffer capacity of the saliva of those subjects who are immune to dental caries may be 40 per cent superior to that of those who are susceptible. Dreizen, Mann, Cline, and Spies[1] proposed a test to evaluate the buffer capacity of the saliva in relation to the dental caries. The test is rational, but the technic is complicated. Therefore, we propose a modification.

METHOD

Three milliliters of saliva stimulated with paraffin is diluted to 6 ml. with distilled water, and 3 drops of chlorophenol red indicator is added.

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The solution has a red-purple color at pH 7. Lactic acid, 0.01 N, is added drop by drop until the indicator turns yellow. The amount of acid added is recorded.

RESULTS

We have obtained good results in the work carried out with this method and have evaluated it in studies on 204 children. These results (Table I) demonstrate the existence of a clear relationship between the concentration of the salivary buffers and the number of cavities. The subjects who were immune to caries had a high buffer power; subjects with rampant caries did not.

TABLE I

GROUP	NUMBER OF CHILDREN	D. M. F.	AVERAGE OF LACTIC ACID (0.01 N CONSUMED)
I	39	0-1	7.5
II	70	2-4	5.7
III	75	5-10	4.3
IV	20	More than 10	3.7

In carrying out studies with this method the results may show an inversion of the relationship between salivary buffers and cavities because of the caries activity.

These results enable us to corroborate that there is a close relationship between the buffer power of saliva and caries activity (Fig. 1) .

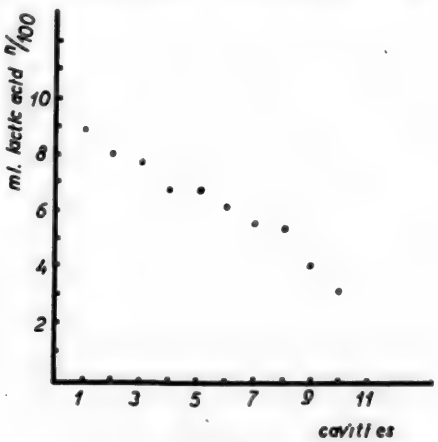

Fig. 1.

DISCUSSION

The susceptibility of an individual to a given disease should be determined before deciding whether preventive measures are needed. The caries-susceptibility tests of Hadley,[6] Fosdick,[2] Stephan,[7] and Snyder[8] have a common defect—they may be applied only by specially trained personnel in

specially equipped laboratories. The test used as a basis for our evaluation[1] does not completely escape this defect—its application requires a good potentiometer and the technic is difficult.

We believe our test is more effective because (1) only a few inexpensive, easily handled laboratory materials are needed, (2) the technic is simple, and (3) the results are reliable. However, the critical pH of 6 is determined by an indicator-colorimetric method, which is not as exact as one determined by a potentiometer. This disadvantage in our test is decreased by the use of dilute acid in the titrations to increase the tolerance.

SUMMARY AND CONCLUSIONS

A method is described to test dental caries activity. The procedure is to determine the buffer power of the saliva with 0.01 N lactic acid and to note a change of the pH from 7 to 6 using chlorophenol red as the indicator.

The results of our experiment with a group of 204 children are given. A large number of cavities corresponds to a low buffer power, and conversely.

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DENTAL FINDINGS IN A NUTRITIONAL STUDY OF SCHOOL CHILDREN IN FIVE GUATEMALAN HIGHLAND VILLAGES

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THERE are few published reports of the incidence of dental caries and other dental conditions from the Central American area. In view of the wide environmental, racial, and dietary variations encountered in this relatively small region, systematic dental studies comparing different groups should contribute to an understanding of etiological factors. Caries has been reported to be exceedingly prevalent in Nicaragua,[1] and a recent survey of 137 families in the rural zone of Turrialba, Costa Rica, revealed a very high DMF rate.[2] The only previous study from Guatemala[3] showed 5.4 DMF teeth per person in children 6 to 14 years of age in Guatemala City.

During nutrition surveys, workers of the Institute of Nutrition of Central America and Panama (INCAP) found the teeth of school children in the highland department of Sacatepéquez, adjacent to Guatemala City, to be relatively good.[4] The present study uses some of these data and by additional examinations confirms this impression. The dental findings are also compared with some of the laboratory, dietary, and other clinical data available on the same children.

MATERIAL AND METHODS

Four hundred forty-two school children from 6 to 14 years of age were examined in five villages near Antigua, Guatemala, in the Department of Sacatepéquez. All of the villages were located between 4,700 and 6,800 feet and were within one hour's drive of Guatemala City. In the diets of these children an average of nearly 65 per cent of the protein and 70 per cent of the calories came from whole corn consumed in the form of tortillas.[5] Since these tortillas are made by a lime process, considerable additional calcium is introduced into the diet. The average daily calcium intake varied from 520 to 810 mg. per child depending on the village. The diets are exceedingly low in vitamin A and riboflavin as well as in animal protein and fat. The intake of calories, thiamin, and niacin appears to be adequate. The height and weight of these children are 2 to 3 years retarded by United States standards.[6-8] Their bone ages, as read from x-ray films of the wrist,[9] are 1 to 2 years behind the standards of Greulich and Pyle for the United States.[10]

With the help of a dental explorer and tongue depressor, each child was examined for the presence of decay and gingival lesions as well as any malformations or malpositions which might be present. As suggested by Blackerby,[11] a decayed tooth was defined as one with a demonstrable area of caries

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on one or more surfaces. Pits and fissures were considered carious only if they could be definitely penetrated by the explorer. None of the children had received dental care so that apart from the normal loss of deciduous teeth, missing and filled teeth were not tabulated. Gingival lesions were noted as present or absent and their type indicated.

Blood samples were drawn from the majority of these children and refrigerated immediately after being taken. They were delivered to the laboratory within 6 hours for aliquoting and freezing. The blood serum of these samples was analyzed by micromethods for total protein,[12] vitamin A and carotene,[13] total tocopherols,[14] alkaline phosphatase,[15] riboflavin,[16] and ascorbic acid.[17, 18] The method for ascorbic acid was modified by using a solution of copper sulfate and thiourea instead of norite as a reducing agent.

RESULTS

In Fig. 1 the total incidence of caries at each age is shown for children in each of the 5 villages. The number of children in each age group in a single village is often too low to justify conclusions regarding differences among villages. However, the children tend to enter school with 4 or 5 carious teeth and leave at age 12 with 1 or 2 carious.

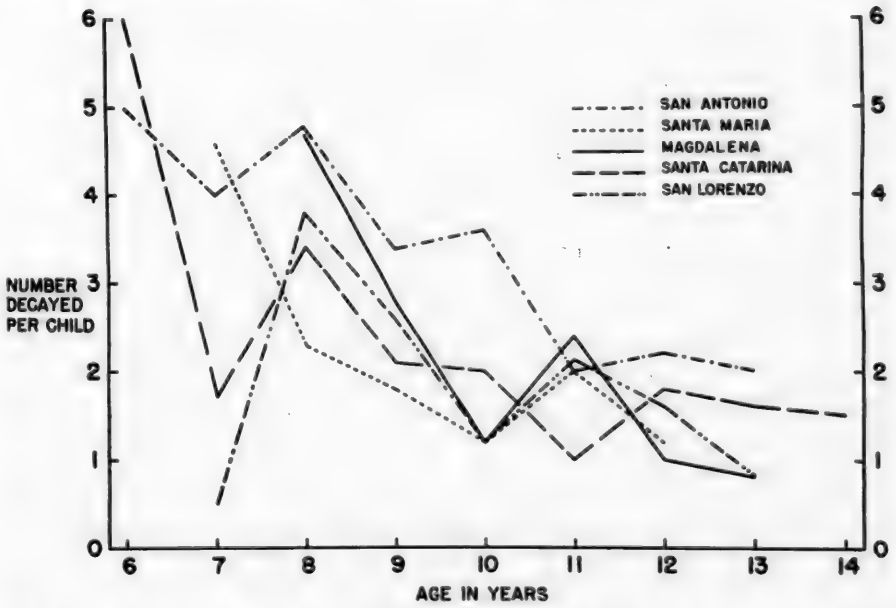

Fig. 1.—Age differences in the incidence of caries in children in five rural Guatemalan villages.

In Fig. 2, the rate of caries in deciduous teeth is shown separately from that in the permanent teeth for children in 3 of the villages. Most of the caries in this age group occurs in the deciduous teeth and, as these are lost,

the total amount of caries decreases, as shown in Fig. 1. However, there is a steady increase in the incidence of caries in the permanent teeth from the time of their first appearance at age 6 to 7, although the total, even at age 13, is less than 2 per child.

The boys appear to have a higher rate of caries in their deciduous teeth and a lower rate in their permanent teeth than the girls. However, the number of children in each group is small and the variation among them considerable so that neither the comparisons by age and sex groups nor the overall comparisons are significant at the 5 per cent level when the chi square test is applied.

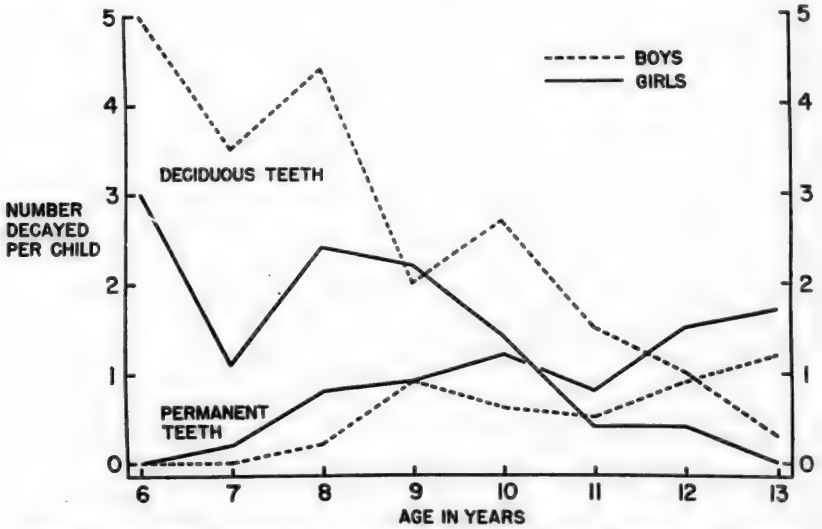

Fig. 2.—Incidence of caries among children of three rural Guatemalan villages.

It is of interest that, at 7 years, one-half of the boys have lost their deciduous incisors and the permanent incisors are beginning to appear, whereas almost all of the girls still have their deciduous incisors at this age. However, at age 12, 41 per cent of the girls have changed all of their teeth and only 26 per cent of the boys have done so. In comparisons with United States standards[19] the eruption of teeth in both boys and girls was found to be retarded at least 1 year.

In Table I the data obtained from examination of the gingivae of the children in all 5 villages are listed together with the number of children in each age group. Nearly all of the cases recorded represent local gingivitis associated primarily with poor dental hygiene. In no age group do more than 10 per cent of the children show gingivitis. No cases were encountered in which systemic disease or nutritional deficiencies were suspected as primary etiological factors.

An attempt was also made to relate serum protein and vitamin levels determined as part of the general nutrition studies of INCAP in these villages

TABLE I. AGE AND DISTRIBUTION OF GINGIVAL LESIONS IN CHILDREN IN FIVE GUATEMALAN HIGHLAND VILLAGES

AGE	NUMBER EXAMINED		GINGIVAL LESION		TOTAL PER CENT
	BOYS	GIRLS	BOYS	GIRLS	
6	1	2	0	0	—
7	14	13	0	1	3.7
8	37	21	4	0	6.8
9	40	38	2	1	3.8
10	45	29	4	1	6.7
11	41	24	1	2	4.6
12	49	31	3	4	8.7
13	34	17	3	1	7.8
14	6	0	1	0	—
Total	267	175	18	10	6.3

to the incidence of gingival and dental lesions. In Table II children free from caries and those with one or more carious teeth are compared in respect to total protein, riboflavin, vitamin C, carotene, vitamin A, vitamin E, and alkaline phosphatase. The levels of each nutrient for each of the 2 groups were compared by means of the t test,[20] but no differences reaching the 5 per cent level of significance were found. Similar comparisons for children with and without gingivitis also failed to reveal significant differences in the blood constituents.

TABLE II. SERUM VALUES IN SCHOOL CHILDREN WITH AND WITHOUT CARIES IN FIVE GUATEMALAN HIGHLAND VILLAGES

		BOYS		GIRLS	
		WITH CARIES	WITHOUT CARIES	WITH CARIES	WITHOUT CARIES
Total protein (Gm. %)	$\bar{x}$	7.27	7.23	7.42	7.43
	s	0.47	0.39	0.46	0.47
	N	76	33	43	34
Free riboflavin (μg %)	$\bar{x}$	1.39	1.68	2.10	1.59
	s	1.07	1.29	1.37	0.65
	N	76	33	43	34
Ascorbic acid (mg. %)	$\bar{x}$	1.32	1.30	1.22	1.23
	s	0.36	0.50	0.36	0.40
	N	76	33	43	34
Carotene (μg %)	$\bar{x}$	111.8	106.3	109.1	106.8
	s	45.5	37.6	56.4	61.7
	N	115	49	63	42
Vitamin A (μg %)	$\bar{x}$	25.2	26.6	24.8	25.6
	s	7.2	8.2	8.5	7.2
	N	111	49	63	42
Vitamin E (mg. %)	$\bar{x}$	0.55	0.54	0.45	0.47
	s	0.26	0.17	0.22	0.32
	N	71	32	34	31
Alkaline phosphatase (u. mm.*)	$\bar{x}$	6.70	5.65	5.95	7.42
	s	3.02	2.60	2.49	3.16
	N	76	33	42	34

*1 millimolar unit = 1.79 Bodansky units.

DISCUSSION

Despite poor dental hygiene and relatively deficient diets, the incidence of dental caries in primary school children in the highland area of Guatemala studied appears to be relatively low. Detailed dietary surveys show the diets of these children to be markedly deficient in vitamin A and animal protein and moderately so in riboflavin. On the other hand, the diets, consisting as they do primarily of whole corn consumed in the form of tortillas, are relatively high in crude fiber and contain little free sugar or refined carbohydrates. Lime is added to the corn before it is soaked to soften it for grinding, and sufficient remains to make a significant contribution to the total calcium content of the diet, which seldom falls below 0.5 Gm.

The diets of children in areas of Central America in which the lime process is not employed are, in general, severely deficient in calcium since the milk consumption throughout the area is very low. This is true for children in Turrialba, Costa Rica, where a high incidence of dental caries was found.[2] Another important difference in the diets of the Costa Rican children is the much higher percentage of calories from refined carbohydrate sources, especially panela, a crude brown sugar which furnished an average of 22 per cent of the calories for families in the rural zone of Turrialba.[21] With the increase in calories derived from sugar and other refined carbohydrates, a decrease in the crude fiber content of the diet occurs. Whether or not these are the important contributory factors to the relatively low incidence of dental caries in the present study compared with reports from other regions cannot be stated with certainty.

As part of the nutrition studies of INCAP, unusually extensive blood studies were carried out for essential serum nutrients. These values are within normal limits for the United States except for the serum vitamin A and carotene levels, which tend to be low, and those for ascorbic acid, which tend to be high. When mean serum levels for total protein, riboflavin, carotene, vitamin A, vitamin E, and alkaline phosphatase, the last as an indicator of vitamin D activity, are compared by means of the t test for children with and without caries, no significant differences are encountered. The number of children is sufficiently large that differences might reasonably have been detected if they had occurred.

An attempt was also made to relate differences in incidence of caries and gingival lesions among the 5 villages to observed differences in the dietary habits. This was not successful because the differences observed among the villages as a result of dental and dietary examinations were not in themselves significant except for a somewhat higher animal protein consumption in Magdalena. This latter did not seem to be reflected in the results of the dental and other examinations. The tendency for sex differences in the caries incidence to occur in the data may be attributable to differences in the dietary and work habits of the girls. Since the former did not prove statistically significant, the matter was not investigated further.

It is possible that differences in the fluorine content of the water supplies had some influence on the survey results. The fluorine content of the waters used in 3 of the villages has been studied with the following results: Santa María, 0.4 ppm, San Antonio 0.5, and Xenacoj 0.7.[22] The same author reported a range of 0.4 to 0.9 for 18 villages in the Department of Sacatepéquez. Dental mottling attributable to fluorosis was not observed and has not been reported in Central America.

It has been claimed by some workers that an inverse relationship exists between the amount of sunshine and susceptibility to dental caries.[10, 23-26] The children of the Guatemalan villages studied are exposed to considerable sunshine since the average number of sunshine hours per year from 1950 to 1953 was 2,400.[27]

It should be noted that the relatively good teeth found in these school children are no indication that the same is true of the adult population. Although the incidence of dental caries in the adults in this area has not been studied, it is apparent to anyone working in the villages that the adult dental status is very poor. This may be due to the neglected accumulated effects of a relatively low rate of caries, differences in dietary and other habits in the adults, or poor dental hygiene in any combination.

SUMMARY

Four hundred forty-two school children, 6 to 14 years of age, in 5 rural highland villages in Guatemala were examined for dental status and gingival lesions as part of a general nutrition study which included dietary surveys, physical examinations, hematologic studies, and serum protein and vitamin determinations. No filled or extracted teeth were found. The incidence of caries in this group averaged 2.4 per person. In deciduous teeth the incidence fell gradually from a high of 5 in boys and 3 in girls at age 6 to 0.3 in boys and 0 in girls at age 13. In permanent teeth the incidence at 13 years was 1.7 for girls and 1.2 for boys. These differences between boys and girls are not statistically significant at the 5 per cent level. Gingival lesions varied from 3.7 per cent at age 7 to 9.8 at age 13 and were almost entirely local in nature due to poor dental hygiene.

The average retardation of over 1 year in eruption of permanent teeth was paralleled by over 1 year's retardation in bone age as compared with United States standards and over 2 years' retardation in height and weight. Malpositions or malformations were observed in only 10 of the children. It is thought that the extremely low percentage of refined carbohydrate in the diet may help to explain the relatively low caries incidence despite obvious deficiencies of vitamin A, riboflavin, and animal protein. No significant difference in the levels of serum protein, vitamin A, carotene, ascorbic acid, free riboflavin, and alkaline phosphatase was observed between children with and without dental caries.

Miguel A. Guzmán furnished the serum vitamin values quoted and rendered valuable assistance in the preparation of the report. Data from the clinical examinations made

by Drs. J. Antonio Muñoz and Fabio Castillo F. are also used. The assistance of other members of the Nutrition Field Team of the Guatemala Ministry of Health and personnel of INCAP is gratefully acknowledged.

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A MEASURE OF THE EFFECTS OF HEREDITY AND ENVIRONMENT ON ERUPTION OF THE DECIDUOUS TEETH

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THAT the sequence of eruption of the deciduous teeth is influenced by hereditary factors has been demonstrated by Ford and Mason[3] and suggested by Robinow, Richards, and Anderson[7] and others. In a recent investigation carried out at the University of Toronto by the present writer, it was noted that in each of 28 sibships included in a sample under observation,[4] the pattern of the eruptive sequence for the deciduous teeth was similar. It was important, therefore, to determine the extent of hereditary factors on this process.

Basic to such a determination was the analysis of the mean eruptive time and its variability in monozygotic and dizygotic twins. For this purpose the dates of eruption of a sample of twins were obtained from the files of Dr. Norma Ford Walker,* who in 1941 started to collect records of eruption of the deciduous teeth of Toronto children. The actual dates of eruption were obtained from parents of twins whom the hospital staffs where the children were born recommended as "intelligent parents." These mothers, who had been instructed that a tooth was considered erupted when it had broken through the gum, were also sent specially constructed forms for noting the date of appearance, which could be confirmed by tapping on the gum with a spoon. Further, the parent was asked to indicate whether the times noted were absolutely accurate, approximately accurate, fairly accurate, or far from accurate. Only absolutely accurate records were finally included in the sample.

Table I lists the results for the 2 types of twins. In this analysis MZ* and DZ* twins were counted as 2 individuals, and only the first 8 teeth to erupt were considered.

VARIABILITY VALUES

Results shown in Table I indicate that the mean eruptive times for the two types of twins are very similar. It may be observed further that the variability is greater for DZ twins for all teeth except the upper lateral incisors. That MZ children show a greater variability in the eruption of these teeth may be related to the fact that in both dentitions the upper second incisors show the greatest amount of morphologic variation and are frequently missing or deformed. Lundstrom[5] was able to show in MZ twins that the standard deviation for the

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*MZ and DZ abbreviations are used throughout for monozygotic and dizygotic twins.

TABLE I

THE MEAN ERUPTIVE TIME IN DAYS AND VARIANCE FOR MONOZYGOTIC AND DIZYGOTIC TWINS

TEETH	MZ N	MZ ♀♀	MZ ♂♂	DZ N	DZ ♀♀	DZ ♂♂	MZ $\bar{x}$	DZ $\bar{x}$	MZ S	DZ S	VARIANCE RATIO	$F_{0.05}$
LLCI	66	40	26	142	64	78	228	231	44	63	$\frac{63^2}{44^2} = 2.05$	1.4
LRCI	66	40	26	141	64	77	232	232	45	46	$\frac{46^2}{45^2} = 1.0$	1.4
ULCI	66	40	26	136	63	73	296	283	63	80	$\frac{80^2}{63^2} = 1.6$	1.4
URCI	64	40	24	134	62	72	296	282	48	52	$\frac{52^2}{48^2} = 1.1$	1.4
ULLI	61	40	21	121	57	64	318	312	75	68	$\frac{75^2}{68^2} = 1.2$	1.4
URLI	56	38	18	120	57	63	319	316	72	55	$\frac{72^2}{55^2} = 1.7$	1.4
LRLI	51	37	14	102	51	51	359	373	59	72	$\frac{72^2}{59^2} = 1.0$	1.4
LLLI	50	36	14	102	52	50	364	371	76	87	$\frac{87^2}{76^2} = 1.3$	1.5

N = number of eruptions; $\bar{x}$ = mean eruptive time; S = standard deviation.

breadth of the permanent left and right upper second incisors was greater than in the population. Hereditary factors governing eruption as well as the form of these teeth might have a variable expressivity, in which case it is possible that even small variations in the internal environment may affect the eruptive time.

TABLE II

A COMPARISON OF THE VARIANCE ABOUT THE MEAN FOR CORRESPONDING TEETH IN MONOZYGOTIC AND DIZYGOTIC TWINS

TEETH	$\bar{x}$	S	N	VARIANCE RATIO	$F_{0.05}$	SIGNIFICANT DIFF.
			Monozygotic twins			
LLCI	228	44	66	$\frac{44^2}{45^2} = 0.96$		None
LRCI	232	45	66			
ULCI	296	63	66	$\frac{63^2}{48^2} = 1.7$	1.6	Some
URCI	296	48	64			
ULLI	318	75	61	$\frac{75^2}{72^2} = 1.1$	1.5	None
URLI	319	72	56			
LLLI	364	76	50	$\frac{76^2}{59^2} = 1.6$	1.6	None
LRLI	359	59	51			
			Dizygotic twins			
LLCI	231	63	142	$\frac{63^2}{46^2} = 1.9$	1.4	Some
LRCI	232	46	141			
ULCI	283	80	136	$\frac{80^2}{52^2} = 2.4$	1.4	Some
URCI	282	52	134			
ULLI	312	68	121	$\frac{68^2}{55^2} = 1.5$	1.4	Some
URLI	316	55	120			
LLLI	371	87	102	$\frac{87^2}{72^2} = 1.5$	1.4	Some
LRLI	373	72	102			

N = number of eruptions; $\bar{x}$ = mean eruptive time; S = standard deviation.

BILATERAL DIFFERENCES

The results listed in Table II show that within each of the 2 groups of twins the mean eruptive time for corresponding teeth is very similar, but the standard

deviation is greater for DZ twins than for MZ. A comparison of the amount of deviation between corresponding teeth involves a consideration of bilateral differences. Fang[1] suggested a method for determining bilateral differences in dermatoglyphics. A similar determination was applied to these data using the eruptive time of left and right lower first incisors. The frequency with which both teeth erupted in the same number of days was charted, and also the frequency with which the eruption of the 2 varied. The number of similar and dissimilar eruptions was then expressed as a percentage and this was regarded as a measure of bilaterality. Results showed that the degree of similarity between left and right lower first incisors of a group of MZ twins was 50 per cent; for the same group of twins the degree of similarity between the lower left first incisor of 1 twin and the lower right first incisor of the cotwin was 45 per cent. In contrast to these values were those for DZ twins, among whom the degree of bilateral similarity between left and right lower first incisors in individuals was 45 per cent; between lower left first incisor in one twin and the lower right first incisor in the cotwin was 13 per cent.

The similarity in the results obtained for the eruption of corresponding teeth in a group of twins and for teeth in the left and right sides of cotwins in MZ pairs can be attributed only to the common genotypes of the pairs. Since the eruptive time of teeth of the left and right sides of MZ cotwins is almost the same as that of corresponding teeth in the same group of individuals, it is obvious that in the case of MZ twins the deviation about the mean eruptive time for corresponding teeth will be similar.*

In the case of twins who have developed from 2 zygotes, there is not the same degree of similarity between the eruptive time of corresponding teeth in a group of individuals, and of left and right sides of cotwins. Therefore, in the case of DZ twins the standard deviation is much greater for corresponding teeth.

A MEASURE OF THE EFFECTS OF HEREDITY AND ENVIRONMENT ON THE TIME TAKEN FOR A TOOTH TO ERUPT

The object of this calculation was to determine the intraclass correlation for MZ and DZ twins. Since MZ twins have the same genotype, the intraclass correlation will be greater for them than for DZ twins. In the case of MZ twins the coefficient of correlation (r) is the result of the following factors: (1) The same main genes for the character in question, (2) some other genes (modifiers), and (3) same environmental factors. In DZ twins the coefficient correlation (r) is due to: (1) some common hereditary factors and (2) common environmental factors. The same environmental factors in MZ twins can be compared with those in DZ twins. Thus, it follows that the amount of effect or variance which is the result of heredity can be estimated, as shown by Newman, Freeman, and Holzinger.[6]

*The sample of eruptive times on which these observations are based may be obtained from the author.

The intraclass correlation was calculated by means of a method of analysis of variance[2] which was as follows:

SOURCE OF VARIANCE	DEGREES OF FREEDOM	MEAN SQUARE
$A = \sigma_w^2 + 2\sigma_f^2 = \Sigma \frac{(x_1 + x_2)^2}{2} - \Sigma^2 \frac{(x_1 + x_2)}{2N}$	$N - 1$	$\frac{A}{N - 1}$
$B = \sigma_w^2 = \Sigma \frac{(x_1 - x_2)^2}{2}$	N	$\frac{B}{N}$

Where:

σ_w^2 = Variance due to individual difference.
σ_f^2 = Variance due to common factors in paired twins.
N = Number of twins.
x_1 = One member of each twin pair.
x_2 = The other member of each twin pair.

The formula for calculating the intraclass correlation is:

$$\rho = \frac{\sigma_f^2}{\sigma_f^2 + \sigma_w^2}$$

The results obtained from applying these statistics are shown in Table III. These give an estimate of the correlation for the 2 types of twins, for sibs, and for random pairs with regard to time taken for a tooth, the lower left first incisor, to erupt. It is clear that the highest correlation is for MZ twins, although that for DZ twins shows some significance. The values for sibs and random pairs are not significant. Considering that sibs have the same parents, one might have expected a value closer to that for DZ twins. The difference here might be due to the small sample.

The next consideration was to determine the hereditability value in this correlation. This determination was possible by using Holzinger's formula, which assumes that environment is similar in paired twins. Common hereditary factors in twins are genes of varying consequence, some affecting the character in question directly, others being of the nature of modifiers.

TABLE III

CORRELATION COEFFICIENTS FOR THE ERUPTIVE TIME OF THE LOWER LEFT CENTRAL INCISOR FOR TWINS, SIBS, AND RANDOM PAIRS

TYPE OF PAIR	NO. OF PAIRS	CORRELATION	STANDARD ERROR
MZ twins	33	0.91	
DZ twins	71	0.59	
Sibs	23	0.16	0.19
Random pairs	46	0.21	0.14

$$\text{S.E.} = \frac{1 - r^2}{\sqrt{N}}$$

Applying Holzinger's formula:

$$h^2 = \frac{r_i - r_f}{1 - r_f}$$

Where:

h^2 = The amount of effect due to heredity.
r_i = The correlation coefficient in uniovular twins.
r_f = The correlation coefficient in binovular twins.

Thus, $h^2 = 0.78$.

It was therefore assumed that the effect of heredity on the time taken for a tooth to erupt is much greater than that of environment. A measure of the effect of heredity was found to be 78 per cent whereas that of environment was only 22 per cent.

SUMMARY

The mean eruptive times of 8 teeth of a group of MZ and DZ twins have been compared.

Similarity in eruptive time between left and right teeth of an individual has been shown to be almost the same as that between left and right teeth of pairs of MZ twins. This was not true for DZ twins.

The intraclass correlation for MZ twins has been estimated. It is 0.91 for MZ twins and 0.56 for DZ twins. The effect of heredity on the eruptive time of a tooth was estimated as being 78 per cent. Heredity, therefore, plays an important part in the time taken for a tooth to erupt.

The author wishes to express her sincere appreciation to Professor Norma Ford Walker for her guidance and criticism throughout the entire investigation, and also to Professor Robert Moyers who provided much valuable criticism in the latter stages of this work. Facilities for this study were provided by the Department of Zoology, University of Toronto, and the writer wishes to thank members of the staff in that Department who assisted her with various aspects of the problem.

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A CLINICAL INVESTIGATION OF THE EFFECTS OF AQUEOUS IODINE SOLUTIONS

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THE clinical effects of several types of dilute aqueous iodine solutions have been investigated. This was done to see whether the differences in chemical composition, antibacterial activity, and toxicity that have been reported by several investigators could be related in any way to clinical observations.

Chemical Nature of Iodine Solutions.—An aqueous solution of elemental or diatomic iodine contains small quantities of other species of iodine. This can be seen from the equations:

$$I_2 + H_2O \rightleftharpoons HIO + H^+ + I^- \quad (1)$$

and

$$I_2 + I^- \rightleftharpoons I_3^- \quad (2)$$

The hydrolysis constant[1] for equation (1) is:

$$K = 3 \times 10^{-13}, \quad (3)$$

and, therefore, if an elemental iodine solution is kept below a pH 6 and near saturation, the diatomic variety of iodine will predominate.

The equilibrium constant for the reaction in equation (2) at 25° C. is

$$K = 7.14 \times 10^2. \quad (4)$$

It is this reaction which is responsible for the practice of adding KI to iodine solution since the maximum solubility of iodine alone in water at room temperature is only 0.03 per cent.

A 0.02 or 0.03 per cent solution of elemental iodine has a pH of about 4 to 5. As can be seen from equations (1) and (2), decreasing the hydrogen ion concentration by buffering the iodine solution at a pH above 6 will appreciably increase the tri-iodide concentration. Equation (1) shows that with the addition of small concentrations of hypoiodus acid, the HIO will suppress the formation of tri-iodide to the vanishing point even in neutral aqueous iodine solutions.

One may estimate the distribution of available iodine between the forms I_2 and I_3^- at any pH for aqueous systems and the effect that hypoiodous acid plays on this distribution in general. This can be done by combining equations (1) and (2), giving

$$K = \frac{(HIO)\ (H^+)\ (I_3^-)}{(I_2)^2} \cong 2 \times 10^{-10} \quad (5)$$

Equation (5) shows that the concentration of tri-iodide is inversely proportional to the hypoiodous acid concentration and increases exponentially

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with the pH. One should also consider, particularly for neutral solutions, the fact that hypoiodous acid is extremely unstable, decomposing irreversibly into iodate (IO_3^-) as an end product in a matter of minutes. According to some,[2] the reaction is,

(6) $$3HIO \rightarrow IO_3^- + 3H^+ + 2I^-.$$

Unless, then, a neutral iodine solution is freshly prepared, it will undergo appreciable changes with time, forming I_3^- and IO_3^- at the expense of the initial concentration of elemental iodine.

Neutral iodine solutions may be stored without appreciable change if potassium or sodium iodide is added to the solution. However, this stability is brought about by the conversion of elemental iodine to tri-iodide according to equation (2), which is unaffected by pH. The widely used Lugol's solution, for example, which consists of potassium iodide added to initially elemental iodine in the ratio of 2:1 will have most of its available iodine in the tri-iodide form. It will be seen, however, from these equilibrium equations, that upon dilution, the tri-iodide complex will dissociate so that at very high dilutions, for example, a few parts per million, the free iodine in an aqueous KI-I_2 system will exist almost entirely as elemental iodine. Thus, at very high dilutions the free iodine of all therapeutic preparations will be practically in the same form.

In general, an aqueous solution made by dissolving crystalline iodine in water at a pH of 6 or 7 will contain the forms I_2, I_3^-, I^-, HIO, and IO_3^-, the amounts of which will depend upon the concentration of available iodine, the presence of potassium iodide, and the age of the solution. If a neutral aqueous solution of 0.02 to 0.03 per cent of free iodine is prepared freshly, almost all the free iodine will be either in the elemental or tri-iodide form, depending upon the quantity of potassium iodide that has been added.

Antibacterial Activity of Iodine.—Although the antibacterial activity of iodine has been recognized for many years, it has only been recently that some light has been thrown on the antibacterial activity of the various common forms of iodine. It is generally believed that both iodide (I^-) and iodate (IO_3^-) have no appreciable antibacterial activity. In 1939, it was noticed that the addition of potassium iodide to an aqueous iodine solution was accompanied by a decrease in the lethal action of the solution toward anthrax spores.[3] The subsequent quantitative investigations of Wyss and Strandskov[2] and Marks[4] have evaluated, in vitro, the relative antibacterial activity of diatomic iodine, tri-iodine, and hypoiodous acid toward the *Bacillus metiens* spore. They have shown that I_3^- has negligible sporicidal activity as compared with elemental iodine, I_2, as calculated by means of equation (4). By keeping the free iodine* constant and varying the concentration of potassium iodide, they were able to demonstrate that the sporicidal activity of an aqueous iodine solution may be made to vary about 100 fold.

To what extent the differences between the action of I_2 and I_3^- apply to other microorganisms, especially those occurring in the oral cavity, is not known. On the basis of recent work[5] in connection with this investigation,

*The free iodine content of a solution is that quantity of iodine that may be assayed directly by means of a thiosulfate titration.

it has been found that in a solution of I_2 and I_3^- the antibacterial activity toward vegetative cells stems almost entirely from the I_2 component. For example, the antibacterial activity of I_3^- against *Staphylococcus aureus* is less than one-twelfth that of I_2.

Experiments[2] with pure HIO solutions at pH 6, prepared by the action of HgO on iodine, indicated the antibacterial activity of HIO to be one-third to one-sixth that of I_2 at equivalent concentration. On a weight basis for iodine, the activity of HIO would be enhanced by a factor of two. In view of the marked instability of HIO and the fact that the antibacterial effect of mixtures HIO and I_2 have not been tried, the presence of HIO in an iodine solution still remains to be evaluated for antibacterial action.

Toxicity of Iodine Solutions.—Whereas the chemical and antibacterial properties of the various forms of iodine are reasonably known, a search of the literature reveals few reports giving clues regarding the relative toxicity of the various species of iodine toward tissue cells. Nyiri and Dubois[6] compared the toxicity of a saturated aqueous solution of elemental iodine containing colloidal iodine with tincture of iodine U.S.P. The comparison was carried out on rabbits by means of intravenous injections as well as oral administration. These investigators found that the colloidal iodine had one-fourth to one-sixth of the local corrosive action exhibited by tincture of iodine U.S.P. on the mucous membrane of the stomach. Since the tincture contains potassium iodide to initial elemental iodine in the ratio of 5:7, there is a high concentration of tri-iodide. However, the comparison between colloidal iodine and the tincture is not strictly valid, since most of the colloidal iodine is in crystalline form and is unable to act except as a reservoir when the elemental iodine is removed from solution. Also, the tincture contained alcohol which was absent in the colloidal preparation.

Ryan, Stone, Ramsey, and Johnston[7] have reported some clinical results comparing the effects of diluted aqueous Lugol's solution and a preparation of aqueous elemental iodine. The former solution contains a ratio of potassium iodide to iodine of 2 to 1. They reported from clinical studies that the elemental iodine preparation showed considerably less toxicity than Lugol's solutions.

In spite of these results, the relative toxicity of the various forms of iodine is yet to be determined on a strictly comparable basis. In a sense, the present clinical investigation may be considered directly related to this problem.

EXPERIMENTAL

Chemical Composition of Iodine Solutions.—The present work was designed to observe the clinical effects of 3 aqueous solutions of iodine as an oral lavage, all solutions having the same concentration of free iodine (0.02 per cent) but varying amounts of potassium iodide. Solution I was diluted Lugol's solution NF. This contained a ratio of iodine to potassium iodide of 1:2. Solution II was diluted strong iodine tincture NF, which contained a ratio of iodine to potassium iodide of 7:5. Solution III was a solution of

diatomic iodine buffered at pH 6, containing a slight excess of hypoiodous acid. Its preparation consisted of using an effervescent tablet of potassium iodide and chloramine-T. The tablet contained enough available chlorine to oxidize the iodide completely to iodine and convert a small quantity of newly formed iodine (0.002 per cent) to the hypoiodous state.* Thus it was possible to use an iodine solution that had a minimum of tri-iodide during its clinical use since any reduced or "spent" iodine would be rapidly reoxidized to the elemental state in the presence of hypoiodous acid.

The concentrations were kept at 25 mg. of free iodine in 4 ounces of water. The KI-chloramine-T tablets contained 25 mg. of available iodine. They were also dissolved in 4 ounces of water immediately before the solution was used. Calculations using equations (1) to (5), spectrophotometric analysis, and thiosulfate titrations revealed the results summarized in Table I.

TABLE I
COMPOSITION OF CLINICAL SOLUTIONS CONTAINING 25 MG. OF FREE IODINE IN 4 OUNCES OF WATER

CHEMICAL FORM OF IODINE	SOL. I	SOL. II	SOL.* III
Diatomic iodine, I_2 mg.	10.5	17.4	21.7
Tri-iodide, I^-_3 mg.	21.8	11.4	0.1
Iodide, I^- mg.	30.9	9.8	0.1
Iodine as hypoiodous acid, HIO mg.	<0.1	<0.1	3.0

*Analysis for this solution refers to 25 mg. of available iodine.

Clinical Phase of the Investigation.—Four hundred and sixty-eight patients, ranging between 15 and 70 years of age, were investigated. The inflammatory lesions studied in this investigation were divided generally on an oral geographic basis: (A) inflammatory lesions of the contiguous soft periodontal tissues; and (B) aphthous and traumatic ulcers of the mucosa of the cheeks, lips, and edentulous areas of the ridges up to, and including, the tissue flexion line.

The lesions described under A were further divided into the following groups:

1. Inflammation limited to the marginal gingivae, not associated with "absolute" periodontal pocket† formation and revealing no radiographic evidence of loss of marginal alveolar bone; predominantly clinical; local aggravating factor: materia alba.

2. Acute necrotizing inflammatory lesions of the gingival tissues attacking the interdental papillae and resulting in a quantitative loss of the soft tissues, revealing wide embrasure spaces. Predominantly clinical; local aggravating factors: calculus, detritus, debris.

3. Marginal periodontitis with "absolute" pocket formation; occlusal disharmonies resulting from either premature loss of teeth and failure to

*Obtained from Heliogen Products, Inc., Long Island City, N. Y.

†"Absolute" periodontal pocket formation refers to a clinical soft-walled pocket between a tooth and contiguous gingiva, the position of the epithelial attachment of the gingiva being at a point on the root surface of the tooth formerly occupied by periodontal membrane.

replace them, or abnormal position of the teeth, either to each other on the arch, or arch to arch (in terms of malocclusion), resulting in occlusal prematurities in the static rest position; horizontal overloading of the teeth in the functional chewing patterns; loss of vertical parallelism of the contacting marginal ridges with plunger cusp and food impaction areas. The predominant clinical local aggravating factor: foreign matter.

It is interesting to note that the irritating factors common to all 3 types of cases described in Group A were foreign matter, bacterial plaque formation, and debris.

The lesions described under B were further divided into two groups:

1. Aphthous ulcers of the oral mucosa.
2. Traumatic ulcers due to physical impingement of removable prosthetic appliances.

The patients classified in the B group were further divided into acute and subacute because of the nature and sequence in healing, as observed clinically, with aphthae and traumatic ulcers.

Controls.—It was desired to leave exposed the gingivae and teeth of only one jaw at a time during the investigation of the cases in Group A. This isolation was accomplished by the following method. An upper or lower model alternately, from accurately recorded impressions, was fabricated for each patient described under Group A.

The model was coated with a fine talcum dust and rubbed until shiny. The powder was used as a separating medium between model and rubber latex. At intervals of 30 minutes, a coat of liquid latex rubber was painted on each model until a thickness of approximately one-eighth inch was obtained. The rubber was permitted to dry for approximately 24 hours and then dusted with talcum. The rubber sleeve was then removed and fitted to the corresponding jaw of the patient. The criterion for an accurate fit was the degree of snugness of the edge of the rubber at the tissue flexion line near the level of the mucobuccal fold.

At this time, Group A was divided into 3 groups. Each group was given one of the 3 aqueous iodine solutions, as mentioned previously, for use as an oral lavage (b.i.d.). The quantity used for each was approximately 4 ounces of solution. Each patient was given the following home care instructions:

1. Upon arising and before retiring, following tooth-brushing, use a "juice" glass of solution for rinsing the mouth.
2. Each patient was instructed to retain each mouthful of solution, forcibly irrigating it over the oral tissues for about 5 seconds before spitting.
3. Each patient was further instructed to take no liquid or solid food or water rinse for approximately one-half hour following the use of the solution.

Those patients using the rubber sleeve were instructed to place the rubber sleeve snugly and securely in place before using the solution, and procedure as previously described was executed. The rubber sleeve was not to be

removed until after the final mouthful of solution was eliminated. The average time for the wearing of the rubber sleeve was clinically checked as being approximately two and one-half minutes.

The patients in Group B of the investigation were handled in a manner similar to those in Group A.

Clinical Procedure.—1. After examination and diagnosis were completed, a preoperative Kodachrome transparency of each patient was taken with controlled lighting so that all the films exposed would be of the same density and give uniform color values.

2. On alternate cases, using either the upper or lower jaw, a thorough prophylaxis and planing and polishing of the exposed tooth surfaces were executed.

3. Each patient was given either Solution I, Solution II, or the tablets used to make Solution III to be used as a lavage. Either the upper or lower jaw was masked with the previously described rubber sleeve.

4. Each alternate patient was instructed to use Solution I, Solution II, or Solution III, as described previously, in the morning and before retiring, after brushing the teeth. This was done at the beginning of the investigation for a period of 2 weeks on all the cases in Group A.

5. Each fifth patient was instructed not to brush the teeth, but, instead, to use the solution prescribed as directed. No other home-care adjunct or procedure was prescribed.

During the course of the procedure, observations for the patient in Group A were recorded at two-week intervals for the first 3 months, and monthly thereafter, with check Kodachrome transparencies made of each case at monthly intervals. The patients in Group B were re-examined at intervals of 24 to 48 hours for a period of 6 days.

Observations were based on the acuteness of the inflammatory process associated with the presence or absence of pain; the degree of gingival enlargement (differentiated as to edematous or hyperplastic changes); and color, tone, and surface texture of the tissues.

EXPERIMENTAL RESULTS

Clinical Observations.—

Group A:

1. Inflammation limited to the marginal gingivae, not associated with absolute pocket formation.

(a) Patients treated and instrumentized for marginal gingivitis, using Solution I or Solution II in conjunction with the operative procedure and as a lavage in home care, adjunctively, revealed no significant inflammatory remission or healing, other than could be attributed to results of the treatment and good oral hygiene, 7 to 10 days on the average. Fetor oris was markedly reduced.

Patients with marginal gingivitis, instrumentized, who used Solution III in conjunction with the operative procedure and as a home-care adjunct, revealed almost total inflammatory remission and healing. Acuteness was

reduced. No pain was originally recorded in this group. Edematous enlargement was reduced. Color, tone, and surface texture of the gingivae were improved. On the average, healing was observed in 4 to 6 days.

(b) In cases of marginal gingivitis, untreated controls, using Solution I or II, adjunctively, in home care, showed little or no improvement in tissue tone, with no reduction in fetor oris. Some patients revealed "wrinkling" of the marginal tissue comparable to the surface reaction of mucous membrane to a mild caustic.

Patients with marginal gingivitis untreated by instrumentation (controls) who used Solution III in home care showed some tissue tone improvement with reduced fetor oris, after 4 to 5 days.

2. Acute necrotizing inflammatory lesions of the gingivae with loss of the interdental papillae, associated with marked pain and fetor oris.

(a) Patients treated for acute necrotizing gingivitis, instrumentized, using Solution I or II in conjunction with the operative procedure and as a home-care adjunct, revealed no greater degree of healing of the ulcers than could be attributed to the clinical treatment and good oral hygiene. Pain and fetor oris were eliminated. Pseudomembrane was no longer clinically demonstrable.

Patients with acute necrotizing gingivitis, instrumentized, who used Solution III in conjunction with the operative procedure and as a home-care adjunct, revealed total clinical inflammatory control with marked abatement of pain and elimination of fetor oris within a period of 24 hours. No pseudomembrane was observed at the second clinic visit, i.e., 2 to 3 days after the initial visit, by which time healing of the ulcers had started.

(b) Patients with necrotizing gingivitis, untreated, who used Solution I or Solution II for adjunctive home care, generally showed no abatement of pain or fetor oris, but rather an increase in the odor and crescendo of the pain. Pseudomembrane was clinically evident.

Patients with necrotizing gingivitis untreated by instrumentation (controls) who used Solution III for adjunctive home care showed abatement of pain in approximately 36 to 48 hours and almost immediate control of the fetor oris. No pseudomembrane was observed after 48 hours; healing of the ulcers had started approximately 24 hours later than the treated areas.

3. Marginal periodontitis associated with "absolute" pocket formation and exudative discharge which appeared to be pus.

(a) Patients treated for marginal periodontitis, instrumentized, who used either Solution I or Solution II as a lavage in conjunction with the operative procedure, and as a lavage adjunct in home care, showed a marked reduction of the inflammation and exudate and a reduction of the absolute pocket over a prolonged period of time, about 3 to 4 weeks, and considerable improvement of tissue tone was noted. Several clinical visits were necessary to effect this result. This is the only group in which Solutions I and II appeared to be beneficial as compared to the controls.

In patients with marginal periodontitis with "absolute" pocket formation, instrumentized, who used Solution III as a lavage in conjunction with the operative procedure and as a home-care adjunct, the inflammatory process was markedly reduced in 8 to 10 days, but the pockets were not reduced. However, abatement of the exudate, which appeared to be pus, was observed.

It would appear that maintenance with Solution III as a lavage in the home-care regime reduces fetor oris, maintains optimum tone of the tissues after clinical treatment has been effected, and produces no visible toxic or undesirable side reaction.

(b) Patients with marginal periodontitis, untreated by instrumentation (controls), who used either Solution I or Solution II adjunctively in home care, revealed no appreciable reduction of the inflammation, amount of exudate, or pocket formation. No improvement in the surface texture or tone of the tissues was clinically demonstrable.

Patients with marginal periodontitis untreated by instrumentation (controls), who used Solution III in home care, revealed some surface tissue tone improvement in 8 to 10 days. No reduction of pocket or exudate was observed.

Group B patients were divided into acute and subacute aphthae and traumatic ulcers:

1. Patients with isolated, acute aphthae using Solution I or Solution II as a lavage, b.i.d., revealed no reduction in pain and the ulcers ran their clinical course in healing over a period varying from 10 days to 2 weeks.

2. Patients with acute traumatic ulcers from removable prostheses revealed no reduction in pain after using Solution I or Solution II with discontinuance of the use of the prosthetic appliance. The ulcers became healed in a period of 2 to 5 days.

3. Patients with subacute aphthae or traumatic ulcers, who used Solution I or Solution II as directed, seemed to have already entered the healing stages. Although they did not present exquisite pain, resolution was not enhanced by the use of either solution as a lavage.

4. Patients with aphthae or traumatic ulcers, who used Solution III as a lavage, showed some reduction of pain in 24 to 48 hours. The healing time was reduced appreciably and was clinically observable coincidental with the loss of pain. All controls showed no visible change in the healing time and required 10 to 14 days as the optimum time for completed repair of the ulcerated lesion.

SUMMARIZED DATA

Four hundred and sixty-eight patients were studied in the course of this investigation, of which a total of 35 are not included in this report. The latter were treated with a solution similar to Solution III which did not contain an overage of chloramine-T. This solution, then, did not contain appreciable quantities of hypoiodous acid. The result of these clinical studies will be the subject of a future publication.

Group A:

1. Marginal gingivitis—168 patients.
   A. Aqueous Iodine Solution I—55 patients.
   B. Aqueous Iodine Solution II—55 patients.
   C. Aqueous Iodine Solution III—58 patients.
2. Necrotizing gingivitis—43 patients.
   A. Aqueous Iodine Solution I—14 patients.
   B. Aqueous Iodine Solution II—15 patients.
   C. Aqueous Iodine Solution III—14 patients.
3. Marginal periodontitis—192 patients.
   A. Aqueous Iodine Solution I—59 patients.
   B. Aqueous Iodine Solution II—65 patients.
   C. Aqueous Iodine Solution III—68 patients.

Group B:

1. Aphthae ulcers—21 patients.
   A. Aqueous Iodine Solution I—5 patients.
   B. Aqueous Iodine Solution II—7 patients.
   C. Aqueous Iodine Solution III—9 patients.
2. Traumatic ulcers—9 patients.
   A. Aqueous Iodine Solution I—3 patients.
   B. Aqueous Iodine Solution II—3 patients.
   C. Aqueous Iodine Solution III—3 patients.

DISCUSSION

This investigation has sought to evaluate clinically the effects of aqueous iodine solutions in general as a therapeutic adjunct in inflammatory lesions of the oral mucosa and to determine the value of such solutions as an adjunct in the home care of patients during periodontal treatment and under periodontal control. Treatment and maintenance of a periodontally involved dentition almost always require that the patient establish and maintain a physiologically clean oral environment during treatment and posttreatment periods. The use of an aqueous solution of iodine offers a possible answer to this problem.

From the results in this work, it appears that an aqueous solution containing a given quantity of free iodine will vary chemically, as well as in its antibacterial and clinical effects. Although the chemical effects of the addition of potassium iodide or hypoiodous acid to an aqueous iodine solution have been known for many years, the medical and dental professions have not differentiated between the wide variety of aqueous iodine solutions that can be prepared.[8] Perhaps this has been due, in part at least, to the lack of clinical evidence to warrant classification of an iodine solution according to its chemical composition. An attempt has been made to correlate the comparative clinical effects resulting from the use of an aqueous solution of diatomic iodine and the widely used aqueous iodine solutions which invariably contain appreciable quantities of iodine in the form of tri-iodide and iodide ions.

It is generally assumed that 0.02 per cent aqueous iodine as used in this investigation is a weak solution.[8] On the basis of free iodine content, such an assumption is obviously correct. However, on the basis of elemental iodine content (i.e., I_2), a 0.02 per cent aqueous solution is two-thirds of the saturation value at room temperature. The question may be raised, what is the maximum possible concentration of elemental iodine in aqueous solution in the presence of the most favorable concentration of potassium iodide? An answer to this question may be arrived at readily by considering an equilibrated aqueous system containing solid iodine. Regardless of how the concentration of the potassium iodide may be varied, the thermodynamic activity of the elemental iodine, i.e., I_2 in solution, must remain constant. The thermodynamic activity is equal to the product of the concentration and its activity coefficient. Since experimental values are not available for the activity coefficient of elemental iodine as a function of added electrolyte (potassium iodide), a theoretical approximation may be made. Unless the concentration of potassium iodide is unusually high, the widely accepted Debye-Hückel theory permits one to set the activity coefficient of elemental iodine to about 1.0 ± 0.1. This small variation is due to the zero charge carried by the diatomic variety of iodine. Thus the maximum concentration of diatomic iodine in water at a given temperature is practically an invariant quantity regardless of the increase in free iodine of the system made possible by the addition of potassium iodide.

The increase of free iodine brought about by the addition of potassium iodide is due to the formation of tri-iodide. In the light of present evidence, fragmentary as it is, the presence of tri-iodide or iodide in an oral lavage may be considered undesirable for the reasons that these forms of iodine have negligible antibacterial activity, whereas their toxicity effects appear to be appreciable.

It should be noted that the elemental iodine solution, Solution III, contained a small quantity of hypoiodous acid. To what extent the observed clinical effects are due to this material or a combination of I_2 and HIO is not clear at this time. The biologic effects of HIO are not too well known. As for mixtures of elemental iodine, I_2 and HIO, we have been unable to find any data in the literature. Hypoiodous acid may be considered the analogue of the chlorine compound which is found in Dakin's solution. In view of the present status of our knowledge of HIO, it should be assumed that the observed clinical effects of Solution III are, in the main, due to the diatomic form of iodine.

The elemental iodine contents of Solutions II and III do not differ very markedly (25 per cent), as can be seen in Table I. However, the 3 mg. of hypoiodous acid can instantaneously reoxidize the reduced form of iodine according to the equation

$$HIO + I^- = I_2 + OH^-.$$

According to this reaction 3 mg. of hypoiodous acid will generate 6 mg. of

elemental iodine and in addition keep the solution free of iodide and tri-iodide ions. On the basis of this reaction, the elemental iodine content of Solutions II and III may be viewed as differing by about 60 per cent.

Bacteriologic and toxicity experiments are in progress to evaluate the effects of HIO alone and in the presence of elemental iodine. Little attention has been given, in the past, to the therapeutic properties of this iodine compound, perhaps because of its instability. In the case of a solution which is to be used as an oral lavage, if the solution is freshly prepared, as was done in this work with Solution III, the compound may prove to have a definite place in the dental armamentarium and for use in the home-care regime as an adjunctive aid. Hypoiodous acid appears to have unlimited solubility in water. It is clear then that, should future antibacterial and toxicity measurements indicate a favorable ratio or therapeutic "index" for HIO, it will be possible to prepare aqueous iodine solutions having the same (or somewhat greater) elemental iodine concentration as in Solution III but containing greater quantities of free iodine without the usual accompanying increase or even presence of tri-iodide or iodide ions.

SUMMARY

1. A systematic attempt has been made to investigate clinically three chemically different types of aqueous ioidne solution having 0.02 per cent free iodine.

2. A special technique has been developed using a rubber latex sleeve which may be employed generally in a differential method for evaluating the relative efficacy of oral lavages.

3. Clinical differences observed for various aqueous iodine solutions appear to be in accord with the antibacterial activity and perhaps toxicity of these solutions toward tissue cells.

4. A solution of diatomic iodine, I_2, with small amounts of hypoiodous acid, HIO, and practically free of tri-iodide, I^-_3, and iodide, I^-, has been found effective and convenient as an oral lavage.

5. The solution given previously appears the most effective of the 3 iodine solutions for purposes of toning the tissues of periodontally involved patients as described in the text.

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A STUDY OF THE NERVE SUPPLY TO THE UPPER ANTERIOR TEETH

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IN REVIEWING the literature great variance was noted in the descriptions given by anatomists of the innervation to the upper anterior teeth. It is apparent that the nerve supply to the maxilla is not as constant as that to the mandible. The controversial point concerning the innervation to the upper anterior teeth has been whether the anterior superior alveolar nerve is the sole source of innervation or whether the nerve supply is received partly from the nasopalatine nerve, or from a combination of the anterior superior alveolar and nasopalatine nerves.

Cook[1] stated that the anterior superior alveolar nerve does not always conform to textbook description and that, in some cases, it would be impossible for this nerve to innervate the maxillary incisors, i.e., in bilateral cleft lip–cleft palate cases. He has asserted that the nasopalatine nerve contributes to the innervation of the upper anterior teeth in normal individuals and that the nasopalatine nerve alone supplies the maxillary incisors in cases of bilateral cleft lip and cleft palate cases.

Phillips and Maxmen[2] and Phillips[3] reported that by depositing anesthetic solution high in the incisive canal profound anesthesia of the pulps of the upper incisors is obtained. They removed more than 2,000 vital pulps from the maxillary incisors using the nasopalatine block injections. From an undisclosed number of dissections they describe an incisive branch arising from the nasopalatine nerve just within the nasal end of the incisive canal and terminating in the pulp of the first incisors.

Gray,[4] in describing the origin, course, and distribution of the anterior superior alveolar nerve, stated that the anterior superior alveolar nerve arises from the infraorbital nerve just before its exit from the infraorbital foramen and descends in a canal in the anterior wall of the maxillary sinus, dividing into branches which supply the incisors and canine teeth.

The objective of this study was to determine the actual source of innervation to the upper anterior teeth. The variations of the nerves in the area were noted together with other pertinent findings.

The data presented in this paper were taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Northwestern University, 1953.

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METHOD

Twenty-six specimens were employed, selected on the basis of the number of upper anterior teeth present and the presence of the nasal septum.

Because the electrolytic form of decalcification is much more rapid, it was selected in preference to employing only an acid reagent. Approximately 6 to 8 weeks are required to decalcify a specimen sufficiently for dissection with the slow form of decalcification as compared to an average of 52 hours by the electrolytic method. The apparatus utilized consisted of a Pyrex glass beaker with a

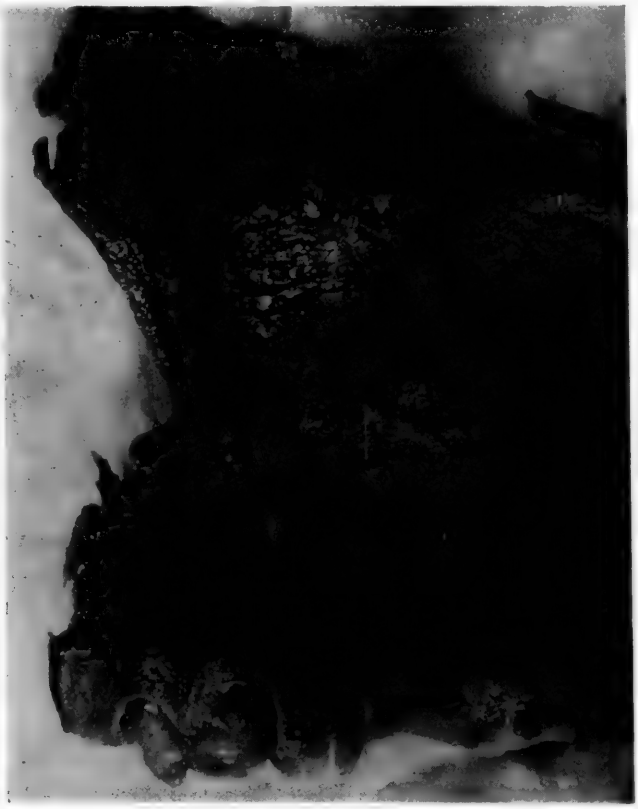

Fig. 1.—There is a dense, complex configuration of nerve filaments arising from the superior dental plexus. The superior dental plexus is formed by the anterior and posterior superior alveolar nerves. There is no middle superior alveolar nerve in this specimen.

platinum electrode, carbon electrode, and a thermometer. The electrodes were connected to a rectifier, which in turn was connected to a 110 A. C. circuit. Richman[5] suggested using an acid solution of 10 per cent by volume of 90 per cent formic acid, and 8 per cent by volume of 37 per cent hydrochloric acid to obtain optimal results. The temperature was maintained below 45° C. to prevent maceration of the tissues. The time required to decalcify the specimens sufficiently for dissection varied from 46 to 65 hours.

Because of the possibility of damaging some of the fine nerve filaments during the dissection procedures, the dissections were commenced in regions where the anterior superior alveolar and the nasopalatine nerves were of greatest diameter and directed toward the areas of innervation.

Fig. 2.—This specimen has 3 main branches which constitute the anterior superior alveolar nerves. The nerves of tactile sensibility are larger in diameter than those innervating the teeth. The probe denotes nerve entering the right first incisor tooth.

OBSERVATIONS

Prior to the decalcification, it was observed that in 42 per cent (11) of the specimens there was no bone covering the apical region of the canine. In the remaining 15 specimens the alveolar plate of bone covering the apical portion of the root of the upper canine was thinner than in a similar position on the apical portion of the other upper anterior teeth. The absence of bone in this particular area in 11 of the specimens may be accounted for by the fact that the specimens employed came from subjects who expired late in life and had subjected their upper canines to a varying degree of attrition and abrasion.

There was a marked inconsistency in the number of branches arising from the infraorbital nerve to form the anterior superior alveolar nerves. The number of these branches varied from 1 to 3. In 31 per cent (8) of the specimens, the origin of the anterior superior alveolar nerve was by a single branch. Forty-six per cent (12) of the specimens had 2 major trunks arising from the infraorbital nerve to originate the anterior superior alveolar nerves. Twenty-three per cent (6) had 3 major trunks arising from the infraorbital nerve forming the anterior superior alveolar nerves.

There is a variance of opinion as to where the anterior superior alveolar nerve arises from the infarorbital nerve in the infraorbital canal. Gray,[4] Jackson,[7] and Schaeffer[8] described the anterior superior alveolar nerve as arising from the infraorbital nerve in the anterior part of the infraorbital canal. Giffen[9] and Prothero[10] stated that it arises about 5 mm. inside the infraorbital foramen. Sicher[11] stated that the origin varies from 6 to 10 mm.

Fig. 3.—The anterior and posterior superior alveolar nerves form the superior dental plexus on the mucous membrane of the maxillary sinus. There is no middle superior alveolar nerve present in this specimen. The nerves diverge from the superior dental plexus to innervate the teeth and the area between the adjacent teeth. Note the number of nerve filaments arising from the superior dental plexus.

posterior to the infraorbital foramen, and according to Brash and Jamieson[12] and Cook,[1] the anterior superior alveolar nerve arises about midway anteroposteriorly in the infraorbital canal.

In this study it was observed that the anterior superior alveolar nerves originate from the lateral or inferolateral aspect of the infraorbital nerve. The distance posterior to the infraorbital foramen, where the anterior superior alveolar nerves arise, appeared to be dependent on the number of branches constituting the anterior superior alveolar nerves. When there was only a single branch forming the anterior superior alveolar nerve, its source of origin was approximately midway anteroposteriorly in the infraorbital canal; in specimens where there were 2 or 3 branches the point of origin of the nearest branch to the infraorbital foramen was in the anterior portion of the infraorbital canal.

After the anterior superior alveolar nerves arise from the infraorbital nerve in the infraorbital canal, they pass laterally to the infraorbital nerve in the canal of Wood or Jones. The nerves descend in bony canals until they reach the anterior aspect of the maxillary bone, where they lie on the mucous membrane of the maxillary sinus. The nerve filaments arising from the main branches of the anterior superior alveolar nerves on the mucous membrane of the maxillary sinus do not assume any definite pattern.

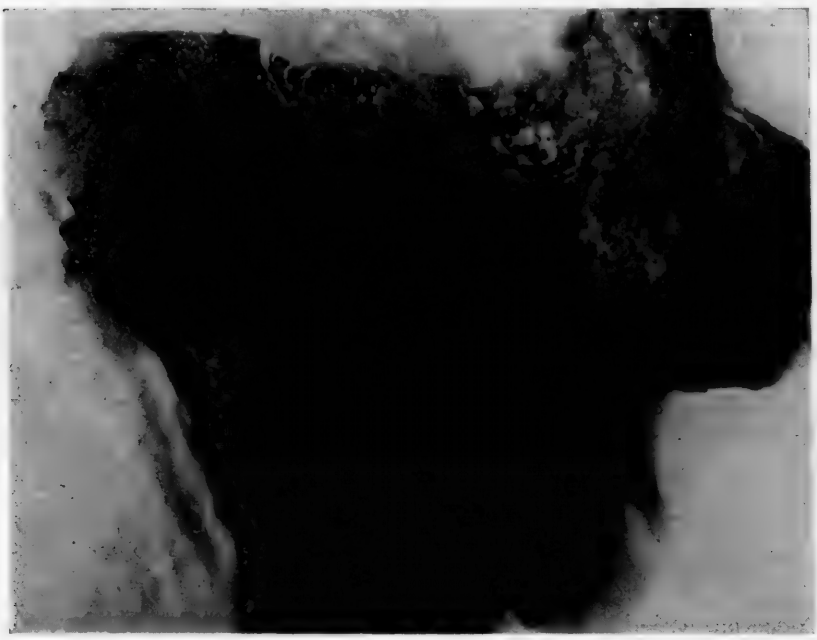

Fig. 4.—This specimen shows the anterior superior alveolar nerve arising midway anteroposteriorly in the infraorbital canal. The middle superior alveolar nerve arises in the posterior portion of the infraorbital canal. Note the superior dental plexus formed by the anterior, middle, and posterior superior alveolar nerves. These nerves lie on the mucous membrane of the maxillary sinus. Observe the nerves innervating the teeth and the area between the adjacent teeth.

The variation in the complexity or sparseness of the nerves arising from the main branches of the anterior superior alveolar nerve was one of the most interesting observations made during the course of this study. The student of anatomy should be aware of the great degree of variation found from one specimen to the next. With an appreciation of the marked degree of variation in the nerve distribution the dentist is better able to understand that his technic in administering local anesthesia may not be at fault when he does not obtain good anesthesia. In 31 per cent (8) of the specimens there was a dense, complex arrangement of nerve filaments arising from the anterior superior alveolar nerves. In 69 per cent (18) of the cases a more sparse pattern was observed.

In 88 per cent (23) of the specimens the nerve filaments found between the adjacent teeth were considerably larger in diameter than those innervating the teeth. This finding is in accord with statements in the literature that the nerves of tactile sensibility (fibers between the adjacent teeth) are of medium size and are moderately myelinated while the pain fibers which innervate the teeth are thinner with less myelin or none at all.

In 53 per cent (14) of the cases dissected there was no middle superior alveolar nerve present. In the absence of this nerve the anterior superior and the posterior superior alveolar nerves formed the superior dental plexus. In instances where the 3 superior dental nerves were present, they formed the superior dental plexus.

In 61 per cent (16) of the maxillae studied a nerve filament was dissected which closely approximated a region where a tooth had been removed prior to death. This observation was made not only in the upper anterior region of the dental arch, but in the premolar area where a tooth had been previously removed. According to Best and Taylor's[13] description of degeneration and regeneration of the nerves, the following explanation is suggested for the phenomenon of the nerve filament being present in the region approximating the apex of a previously extracted tooth: There is a retrograde degeneration of the neuron on the proximal side of the apex of the tooth removed. The nerve fiber as far centrally as the first node of Ranvier shows changes of degeneration. Regeneration of the nerve fiber gradually occurs by a downward growth of the neurofibrils from the proximal portion of the severed nerve.

It was observed during the course of study that 26 per cent (7) of the specimens had branches arising from the nasopalatine nerve at the nasal end of the incisive canal. This nerve filament did not enter the apex of the first incisor tooth in any of the specimens in this study, but merely approximated the apical region of the first incisor tooth. Phillips and Maxmen[2] and Phillips[3] described an incisive branch of the nasopalatine nerve which courses through a fine canal before it enters the apex of the first incisor tooth. The prevailing belief in a supplementary nerve supply to the first incisors by the nasopalatine nerves is probably based on the fact that by giving a nasopalatine nerve injection in the incisive canal complete anesthesia of the incisors is obtained. This seems to be a logical conclusion considering that the nasopalatine nerve courses through the incisive canal. However, upon close anatomic observation of a skull it is noted that in placing the needle of the syringe in the incisive canal it closely approximates the apex of the maxillary first incisor tooth. The bone is quite cancellous in this area so that regardless of the nerve supply to the incisors there would be a diffusion of the anesthetic solution in the area of the apices of the teeth with resultant anesthesia. In cases of bilateral cleft lip and cleft palate specimens, it could be assumed that the nasopalatine nerve would be the logical source of innervation.

In the maxillae in this study it was apparent that nerve filaments arising from the anterior superior alveolar nerves innervated the upper anterior teeth. It was possible to dissect the nerves arising from the anterior superior alveolar

nerves into the apices of the teeth in 88 per cent (23) of the maxillae dissected. In the other 3 maxillae it was not possible to trace the nerve into the apices of the teeth due to inadequate decalcification.

CONCLUSIONS

1. The anterior superior alveolar nerve arises from the lateral or inferolateral aspect of the infraorbital nerve.

2. The point of origin of the anterior superior alveolar nerve from the infraorbital nerve in the infraorbital canal varies from the anterior portion of the canal to midway anteroposteriorly in the canal. The point of origin is dependent on the number of branches arising from the infraorbital nerve to form the anterior superior alveolar nerves.

3. The number of branches arising from the infraorbital nerve to constitute the anterior superior alveolar nerves varies from 1 to 3 in number: (a) 31 per cent (8) of the specimens had a single branch arising from the infraorbital nerve; (b) 46 per cent (12) had 2 branches arising from the infraorbital nerve; (c) 23 per cent (6) had three branches arising from the infraorbital nerve.

4. The anterior superior alveolar nerve, after its origin from the infraorbital nerve, passes in a bony canal (Wood or Jones) until it reaches the anterior aspect of the maxillary bone.

5. The nerves arising from the anterior superior alveolar nerves on the anterior aspect of the maxillary bone show a great degree of variation. This variation ranges from a sparseness of nerve filaments to a very dense, complex configuration of nerves with no definite pattern.

6. The diameter of the nerves innervating the teeth is smaller than those of tactile sensibility innervating the mucous membrane and interseptal tissue.

7. There is a close proximity of the nasopalatine nerves and filaments arising from the nasopalatine nerve to the apices of the maxillary first incisor teeth and the close association of filaments of the anterior superior alveolar nerves with the nasopalatine nerves.

8. The middle superior alveolar nerve was absent in over one-half the cases observed (53 per cent). When the middle superior alveolar nerve is absent, the superior dental plexus is formed by the anterior and posterior superior alveolar nerves. In cases where the middle superior alveolar nerve is present the plexus is formed by the anterior, middle, and posterior superior alveolar nerves.

9. The nerve closely approximates the area of the apices of the teeth that have been removed prior to the time the subject had expired.

10. The nerve supply to the upper anterior teeth is derived from filaments arising from the anterior superior alveolar nerves or filaments from the superior dental plexus.

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THE LATIN SQUARE DESIGN IN CLINICAL EXPERIMENTATION

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AS EXPERIMENTATION has progressed in dental research over the years, more and more emphasis has been placed upon the refinement of technical procedures. By the same token, the design of experiments should be improved so as to make them more efficient to enable the investigator to draw sounder conclusions. It is the purpose of this paper to present and explain the clinical application of one such experimental design, the Latin square. This design was introduced by Fisher[1] in order to control 2 factors while studying the effect of a third factor, such as treatment.

An opportunity to use this type of design occurred in an experiment in which the effects of 4 drugs advocated for relaxing cerebral palsy children for operative dentistry were to be assessed.[2] The drugs (treatments) were: A—placebo, B—methylparafynol (Dormison), C—mephenesin (Tolserol), D—pentobarbital sodium (Nembutal), and E—meperidine hydrochloride (Demerol), all administered according to the standard dose per kilogram of body weight used in the institution in which the study was conducted. The drugs were dispensed by a nurse at a given time before operative dentistry was to begin, so that the dental operators did not know what medication was given. The vehicles of all the 5 treatments were similar so that the subjects would not be conditioned in their reactions by the physical nature of the medications employed. In order to control the expected individual variation in cooperativeness, each patient was given one of the drugs for the different operative occasions.

In this study, 2 dentists were employed, one operating and one assisting. The operative procedures were fairly standardized. After the operative session, each dentist would score the patient's cooperativeness without the knowledge of the other dentist and also without the knowledge of how the patient was scored on preceding occasions. The scores went from 1 to 4, with 1 designating the greatest cooperativeness and 4 the least. An attempt had previously been made to adjust the scoring scale so that the intervals between the scores referred to equal changes in cooperativeness.

At the conclusion of the study, a very close approximation of the scores was noted for the 2 dentists. In all cases, however, the score of the operator was used. In order to balance the effects of the time of day during which the work was performed, the various appointments of each patient were scheduled at different times of the day. This factor was not controlled, however, and therefore this effect cannot be analyzed. In addition, the roles of operator and

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assistant were reversed for each group of 5 children. There were 20 boys and 5 girls in this study, with ages ranging from 4 to 13 years. All the girls were studied in one group (III).

Because of a possible "learning effect" in cooperativeness from one operation to the next, the treatments should not be given in the same order for each individual. While this "learning effect" may be eliminated by randomizing the order of treatments for each individual, it may actually be controlled and its effects measured by resorting to the Latin square. In this design, the effects of the variation between patients can be assessed and will enable one to make a more precise comparison between treatments.

TABLE I
STANDARD 5 BY 5 LATIN SQUARE DESIGN

		COLUMNS				
		ORDER OF ADMINISTRATION				
	PATIENT NO.	1	2	3	4	5
ROWS	1	A	B	C	D	E
	2	B	C	D	E	A
	3	C	D	E	A	B
	4	D	E	A	B	C
	5	E	A	B	C	D

When there are only 5 treatments (that is, drugs) involved, a Latin square will be restricted to 5 patients. The treatments are assigned in such a way that each treatment is given only once in a given order. A standard form is

TABLE II
FIVE LATIN SQUARE DESIGNS EMPLOYED IN THIS STUDY

	PATIENT NO.	ORDER OF ADMINISTRATION				
		1	2	3	4	5
Group I	1	B	E	D	A	C
	2	A	D	C	E	B
	3	E	A	B	C	D
	4	D	C	E	B	A
	5	C	B	A	D	E
Group II	6	E	D	B	C	A
	7	D	C	A	B	E
	8	A	B	E	D	C
	9	C	E	D	A	B
	10	B	A	C	E	D
Group III	11	C	E	D	A	B
	12	A	B	E	D	C
	13	E	D	B	C	A
	14	B	A	C	E	D
	15	D	C	A	B	E
Group IV	16	A	C	B	E	D
	17	B	A	D	C	E
	18	E	B	A	D	C
	19	D	E	C	B	A
	20	C	D	E	A	B
Group V	21	D	A	B	C	E
	22	C	E	D	B	A
	23	A	B	E	D	C
	24	E	D	C	A	B
	25	B	C	A	E	D

indicated in Table I. The order of administration is denoted as 1, 2, 3, 4, and 5, the treatments as A, B, C, D, and E, and the patients by their initials or by code numbers. It will be noted that each treatment occurs only once in each column and each row. It is obvious that the number of different treatments must be the same as the number of different patients for each square. For an actual experiment, a standard square as depicted in Table I is arranged randomly. In order to utilize a larger number of subjects, randomized repetitions of the Latin square can be constructed. Thus, in this experiment 25 patients were studied necessitating 5 repetitions of the Latin square. The 5 Latin squares utilized in this study appear in Table II. The data from each Latin square (group) appear in Table III.

TABLE III

PATIENT COOPERATION SCORES FOR 5 GROUPS

	GROUP I					
	ORDER OF ADMINISTRATION					
PATIENT NO.	1	2	3	4	5	TOTAL
1	2	2	2	2	1	9
2	2	2	2	2	4	12
3	2	2	2	2	2	10
4	2	2	2	2	2	10
5	3	2	2	2	2	11
Total	11	10	10	10	11	52

Drug Scores

A	B	C	D	E	Total
10	12	10	10	10	52

	GROUP II					
	ORDER OF ADMINISTRATION					
PATIENT NO.	1	2	3	4	5	TOTAL
6	2	2	2	4	4	14
7	2	2	1	2	2	9
8	2	2	3	1	3	11
9	3	3	2	2	2	12
10	3	4	3	3	2	15
Total	12	13	11	12	13	61

Drug Scores

A	B	C	D	E	Total
13	11	15	9	13	61

The total scores obtained by the 25 individuals for each of the 5 treatments (and for each of the five orders), as well as the distribution of the scores, are given in Table IV. It is immediately apparent from the χ^2 values that there are no significant differences either as to order or as to treatment (at 12 degrees of freedom, P — 5 per cent at χ^2 — 21.026).

The data obtained in the Latin square type of design are usually analyzed by the analysis of variance,[3] if they are measurements fulfilling certain conditions.[4] While these conditions are not completely met by the scoring system

of recording the data, the analysis of variance may be performed as an approximate type of solution. It has been employed successfully in other situations where scores were used.[5] In order to illustrate the calculations and the variety of significance tests possible, the data in Table III will be analyzed by the analysis of variance. For the data of Group III, the following analysis is obtained:

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Between orders	4	1.36	0.34
Between patients	4	14.96	3.74
Between drugs (treatments)	4	0.56	0.14
Interaction	12	2.88	0.24
Total	24	19.76	

	GROUP III					
	ORDER OF ADMINISTRATION					
PATIENT NO.	1	2	3	4	5	TOTAL
11	3	3	3	4	2	15
12	1	2	3	3	2	11
13	2	2	2	2	2	10
14	2	2	2	2	2	10
15	4	4	4	4	4	20
Totals	12	13	14	15	12	66

Drug Scores

A	B	C	D	E	Total
13	12	13	14	14	66

	GROUP IV					
	ORDER OF ADMINISTRATION					
PATIENT NO.	1	2	3	4	5	TOTAL
16	4	4	3	3	3	17
17	4	4	4	4	4	20
18	2	2	2	1	1	8
19	3	3	3	4	4	17
20	2	2	1	1	1	7
Totals	15	15	13	13	13	69

Drug Scores

A	B	C	D	E	Total
15	14	14	13	13	69

	GROUP V					
	ORDER OF ADMINISTRATION					
PATIENT NO.	1	2	3	4	5	TOTAL
21	1	1	2	1	2	7
22	3	2	2	2	2	11
23	3	2	2	3	3	13
24	4	4	4	4	4	20
25	3	3	3	3	2	14
Totals	14	12	13	13	13	65

Drug Scores

A	B	C	D	E	Total
13	13	14	12	13	65

TABLE IV

SUMMARY TABLES OF PATIENT SCORES BY ORDER OF ADMINISTRATION AND BY TREATMENT

	ORDER					
SCORE	(1)	(2)	(3)	(4)	(5)	
1	2	1	2	4	3	
2	11	15	13	10	13	$\chi^2 = 8.00$
3	8	4	7	5	3	$d = 12$
4	4	5	3	6	6	
Total patients	25	25	25	25	25	
Total score	64	63	61	63	62	

	TREATMENT (DRUG)					
SCORE	A	B	C	D	E	
1	4	1	3	3	1	
2	11	16	8	14	13	$\chi^2 = 16.30$
3	2	3	9	5	8	$d = 12$
4	8	5	5	3	3	
Total patients	25	25	25	25	25	
Total score	64	62	66	58	63	

The total degrees of freedom are the number of readings less one $(25 - 1)$; the degrees of freedom for drugs are the number of drugs less one $(5 - 1)$; for patients, the number of patients less one $(5 - 1)$; and for orders, the number of orders less one $(5 - 1)$.

The sums of squares (SS) can be obtained as follows:

SS Between Orders.—Add the squares of the column (order) totals and divide by the number of readings per column. From this, subtract the square of the grand total divided by the total number of readings in the square. This latter term is known as the "correction term" (C.T.). Thus,

$$\frac{(12)^2 + (13)^2 + (14)^2 + (15)^2 + (12)^2}{5} - \frac{(66)^2}{25} = 1.36.$$

SS Between Patients.—Add the squares of the row (patient) totals and divide by the number of readings per row. From this subtract the C.T. Thus,

$$\frac{(15)^2 + (11)^2 + (10)^2 + (10)^2 + (20)^2}{5} - \frac{(66)^2}{25} = 14.96.$$

SS Between Drugs (Treatments).—Add the squares of the drug (treatment) totals and divide by the number of readings per drug. From this subtract the C.T. Thus,

$$\frac{(13)^2 + (12)^2 + (13)^2 + (14)^2 + (14)^2}{5} - \frac{(66)^2}{25} = 0.56.$$

Total SS.—Add the square of each of the 25 individual readings and subtract the C.T. Thus,

$$(3)^2 + (1)^2 + (2)^2 + (2)^2 + \ldots (2)^2 + (2)^2 + (4)^2 - \frac{(66)^2}{25} = 19.76.$$

SS Interaction (Error).—Subtract the SS for Orders, Patients, and Drugs from the total SS. Thus, $19.76 - (1.36 + 14.96 + 0.56) = 2.88$.

On each square (group), tests of significance may be made for Orders (F_1), Patients (F_2), and Drugs (F_3), as follows:

$$F_1 = \frac{\text{Mean Square Between Orders}}{\text{Mean Square Interaction}} = \frac{0.34}{0.24} = 1.42 \text{ at } d_1 = 4 \text{ and } d_2 = 12$$

$$F_2 = \frac{\text{Mean Square Between Patients}}{\text{Mean Square Interaction}} = \frac{3.74}{0.24} = 15.58, \text{ at } d_1 = 4 \text{ and } d_2 = 12$$

$$F_3 = \frac{\text{Mean Square Between Drugs}}{\text{Mean Square Interaction}} = \frac{0.14}{0.24} = 0.58, \text{ at } d_1 = 4 \text{ and } d_2 = 12.$$

Examination of standard tables of F[6], the variance ratio, shows at $d_1 = 4$ and $d_2 = 12$, that $F_{5\%} = 3.26$ and $F_{1\%} = 5.41$. Thus, only the F_2 test could be called "statistically significant." It can be concluded from this analysis that neither the order of operative occasions (F_1) nor the drugs themselves (F_3) produced any significant difference in the patients' cooperativeness under operative dental procedures. On the other hand, patients do differ among themselves in their cooperativeness for dental procedures (F_2). The sums of squares for the analyses of variance for each of the 5 different patient groups appear in Table V. The results are similar to those found for Group III.

TABLE V

SUMS OF SQUARES FOR 5 GROUPS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES				
		I	II	III	IV	V
Between orders	4	0.24	0.56	1.36	0.96	0.40
Between patients	4	1.04	4.56	14.96	27.76	18.00
Between drugs	4	0.64	4.16	0.56	0.56	0.40
Interaction	12	3.92	6.88	2.88	3.28	3.20
Total	24	5.84	16.16	19.76	32.56	22.00

These 5 separate analyses can be combined to give an over-all analysis in which the results of the experiment can be assessed for all 25 patients. The first step in this analysis would be to add the sums of squares of the sources of variation for each of the 5 groups. Thus:

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES
Between patients specific for group	20	66.32
Between orders specific for group	20	3.52
Between drugs specific for group	20	6.32
Interaction specific for group	60	20.16
Total	120	96.14

The total Sum of Squares for the 5 analyses has 120 degrees of freedom, whereas the 125 readings in the 5 Latin squares (groups) have 124. The difference of 4 degrees of freedom measures one more source of variation, Between Groups. Since the 5 orders of operative occasions are the same for all groups,

the source of variation due to Between Orders Specific for Group can be broken down into 2 parts, Between Orders with 5 – 1 = 4 degrees of freedom and Order-Group Interaction with 16 – 4 = 12 degrees of freedom. Similarly, Between Drugs Specific for Group can be broken down into 2 separate sources of variation.

The calculation of the sum of squares of these new terms can be performed as follows (see total scores, Tables III and IV):

$$\text{SS Between Orders} = \frac{(64)^2 + (63)^2 + (61)^2 + (63)^2 + (62)^2}{25} - \frac{(52 + 61 + 66 + 69 + 65)^2}{5 \times 25} = 783.96 - 783.75 \text{ (Correction Term)} = 0.21$$

$$\text{SS Order-Group Interaction} = \text{SS Between Orders Specific for Group} - \text{SS Between Orders} = 3.52 - 0.21 = 3.31$$

$$\text{SS Between Drugs} = \frac{(64)^2 + (62)^2 + (66)^2 + (58)^2 + (63)^2}{5} - \text{C.T.} = 785.16 - 783.75 = 1.41$$

$$\text{SS Drug-Group Interaction} = \text{SS Between Drugs Specific for Group} - \text{SS Between Drugs} = 6.32 - 1.41 = 4.91$$

$$\text{SS Between Groups (see Table III)} = \frac{(52)^2 + (61)^2 + (66)^2 + (69)^2 + (65)^2}{5} - \text{C.T.} = 790.68 - 783.75 = 6.93$$

The completed analysis of variance for all 5 Latin squares considered together would then be:

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Between patients specific for group	20	66.32	3.32
Between orders	4	0.21	0.05
Order-group interaction	16	3.31	0.21
Between drugs	4	1.41	0.35
Drug-group interaction	16	4.91	0.31
Between groups	4	6.93	1.73
Error (interaction specific for group)	60	20.16	0.34
Total	124	103.25	

The various F tests which could then be performed would be:

$$F_1 = \frac{\text{Mean Square Between Groups}}{\text{Mean Square Between Patients Specific for Group}} = \frac{1.73}{3.32} = 0.52$$

at $d_1 = 4$ and $d_2 = 20$.

Since F is less than one* it can be concluded that the 5 groups of patients in this study are not at significantly different levels of patient cooperation.

$$F_2 = \frac{\text{Mean Square Between Orders}}{\text{Mean Square Error}} = \frac{0.05}{0.34} = 0.15 \text{ at } d_1 = 4 \text{ and } d_2 = 60.$$

*Whenever F is less than one in this type of test, it is statistically not significant and the tables of F need not be consulted.

It can be concluded that the different orders of operative occasions do not differ significantly from each other.

$$F_3 = \frac{\text{Mean Square Order-Group Interaction}}{\text{Mean Square Error}} = \frac{0.21}{0.34} = 0.62 \text{ at } d_1 = 16 \text{ and } d_2 = 60.$$

It can be concluded that whatever difference or lack of difference there may be resulting from the different orders of operative occasions may be the same in all five repetitions of the experiment.

$$F_4 = \frac{\text{Mean Square Between Drugs}}{\text{Mean Square Error}} = \frac{0.35}{0.34} = 1.05 \text{ at } d_1 = 4 \text{ and } d_2 = 60.$$

Since $F_{5\%} = 2.52$ and $F_{1\%} = 3.65$ at $d_1 = 4$ and $d_2 = 60$, it can be concluded that the effects of the different drugs do not differ significantly from each other when all five groups are pooled together.

$$F_5 = \frac{\text{Mean Square Drug-Group Interaction}}{\text{Mean Square Error}} = \frac{0.31}{0.34} = 0.92 \text{ at } d_1 = 16 \text{ and } d_2 = 60.$$

Whatever difference or lack of difference there may be due to the effects of the different drugs given may be the same in all 5 repetitions of the experiment.

It can thus be seen that the drugs employed in this study had no significant effect upon the cooperativeness of cerebral palsy cases in operative dental procedures, and that the different orders of operative occasions likewise had no significant effect upon the results. As was to be expected, the patients did differ significantly with respect to their cooperativeness.

SUMMARY

The Latin square has been offered as a design for clinical experimentation. The analysis of the results for individual squares and for the whole study has been explained and illustrated with data obtained from a study of the effects of different drugs upon the cooperativeness of 25 cerebral palsy children undergoing operative dental procedures.

The author wishes to express his sincere thanks to John W. Fertig, Professor of Biostatistics, School of Public Health of the Faculty of Medicine, Columbia University, for his assistance in the planning and editing of this paper.

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EXPERIMENTAL CAVITY PREPARATIONS IN THE MOLAR OF THE RAT

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INTRODUCTION

THE primary purpose of this investigation was to develop and standardize an experimental method of preparing dentinal cavities in the molar of the rat which could be successfully sealed with a filling material. The effects of such materials upon the pulp could thus be tested.

It is essential to develop methods of testing the effects of filling material and medicaments upon the pulp. A number of experimental studies on the effect of various materials upon the pulp have been investigated on the teeth of the dog, monkey, and man. Human teeth would be the most desirable test objects, but for obvious reasons it is difficult to find healthy human teeth which could be used in experimental investigations of this kind. Monkeys and dogs, while available in sufficient numbers for experimental study on their teeth, are expensive to keep and require considerable attention. The rat, on the other hand, is a convenient and less expensive animal for experimental investigations. The molar of the rat is anatomically and biologically similar to the human molar (Schour and Massler[3]) and therefore well suited for studies of the dental pulp.

A careful study of the literature shows no previous investigation on the rat molar as a biologic testing tool for the effects of dental materials upon the pulp.

METHOD OF CAVITY PREPARATION

The technical difficulties that had to be solved before the rat molar could be employed as an experimental tooth for cavity preparation and subsequent filling were many. They were derived from the following factors: (1) the small size of the molars of the rat, (2) the anatomic position of the molars posterior to an extensive diastema between them and the incisors, making operative accessibility difficult, (3) the difficulty of attaining complete immobilization of the experimental animal in spite of deep anesthesia, (4) the unavailability of drills small enough for operative procedures in the molars of the rat, (5) the necessity to operate under a dissecting microscope, (6) the importance of the proper positioning of the head in view of the anatomic relations of the oral pharynx in the rat.

This investigation was carried out under Contract No. W-DA-49-007-MD 140, with the Medical Research and Development Board, Office of the Surgeon General of the United States Army. It is part of a dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science in Anatomy, University of Illinois, College of Dentistry.

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The maxillary first molar (Fig. 5, *a*) seemed to offer the greatest potentiality for success because of its relative size, position, and accessibility. It therefore was selected as the experimental tooth, while the corresponding first molar on the opposite maxilla served as the control. Rats of approximately 50 days of age were used.

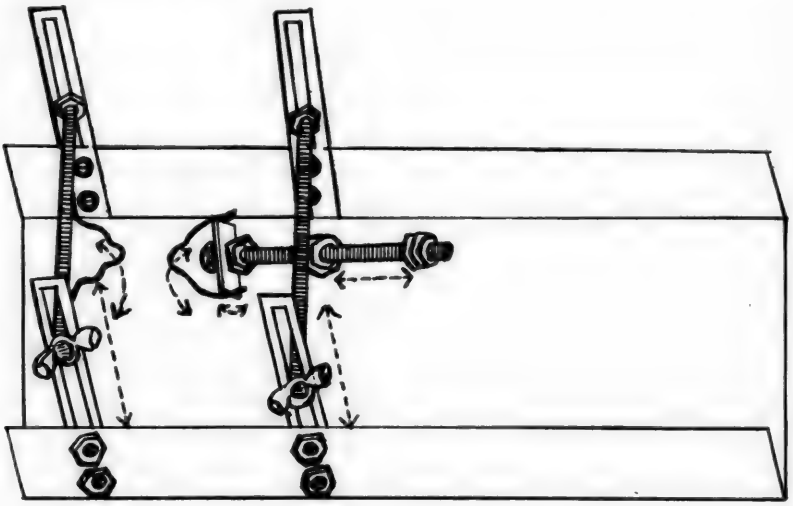

Fig. 1.—Diagram of operating tray with arrows to indicate possible movements of adjustable parts. Note the flexibility of adjustments which can be made.

Site of Experimental Cavity and Instrumentation.—The first attempts at cavity preparations were made with a No. ½ round dental bur, the smallest manufactured size available. The cavities made by the bur proved to be too large in relation to the size of the tooth. Furthermore, because of the narrow deep grooves and high cusps, all too frequently the pulpal horns were exposed. A special drill of smaller dimensions was then made by grinding off the cutting blades from the head of a No. 33½ inverted cone dental bur (Fig. 4, *c*) and in their place grinding 4 planes by means of a rectangular India stone. Two of the planes were made larger and converging. The drills so produced resembled a bibeveled chisel (Fig. 4, *a* and *b*). The dimension of the width of the blade was 0.4 mm. and the length of the head 0.5 mm.

A study of ground sections of rat molars revealed that pulp exposures were least likely to occur if the cavity was prepared on one of the axial surfaces. After a number of trials, the mesial surface of the maxillary right first molar just above the marginal gingiva was chosen as the site most favorable for cavity preparation. The difficulty encountered in starting the preparation in the enamel with the drill was eliminated by using the tip of a No. 1 Kerr Dental Manufacturing Company diamond point (Fig. 4, *d*) for first penetrating the enamel. The cavity could then be easily finished by using the special steel drill.

Operating Table.—An operating table used by Orland[2] in conducting his caries studies on the white rat was of considerable help in perfecting the mechanical design of an apparatus illustrated in Figs. 1 and 3. This special operating table completely immobilized the head and also kept the mouth open to whatever width was desired. A weighted wire cheek retractor, which was

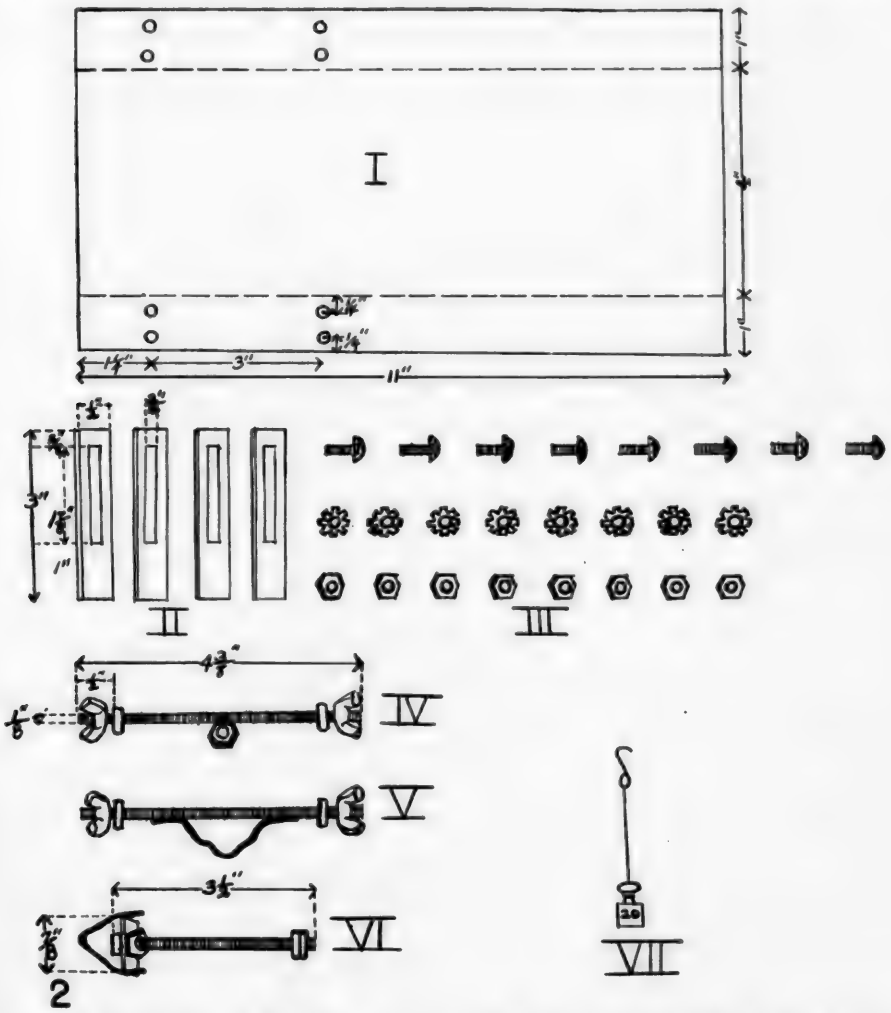

Fig. 2.—Diagram of the parts of the operating table. *I*, Rectangular piece of sheet metal 6" x 11" x 1/8". *II*, Four brass bars 3" x 1/2" x 1/16". Each bar has a slot section 1 7/8" x 3/16" cut out as illustrated. *III*, Eight small brass nuts and bolts with lock washers to prevent loosening of nuts. *IV*, Mandibular crossbar consisting of a 4 3/8" long and 1/8" diameter brass rod. Two brass nuts are soldered to the rod 1/2" from the end. Two brass butterfly adjustment nuts are screwed into each end of the rod. In the center of the rod is soldered a brass nut which will accommodate a 1/8" diameter bolt. *V*, Maxilla-fixing crossbar with looped brass wire soldered to the rod. *VI*, Mandible-retracting rod consisting of a 3 1/2" x 1/8" threaded brass rod with a brass nut soldered at one end. The nut holds a freely moving bar 7/8" long which has a brass wire loop soldered to it. The wire loop engages the mandibular incisors. *VII*, Cheek retractor consisting of a wire loop with a 20 gram weight (for traction) tied to it.

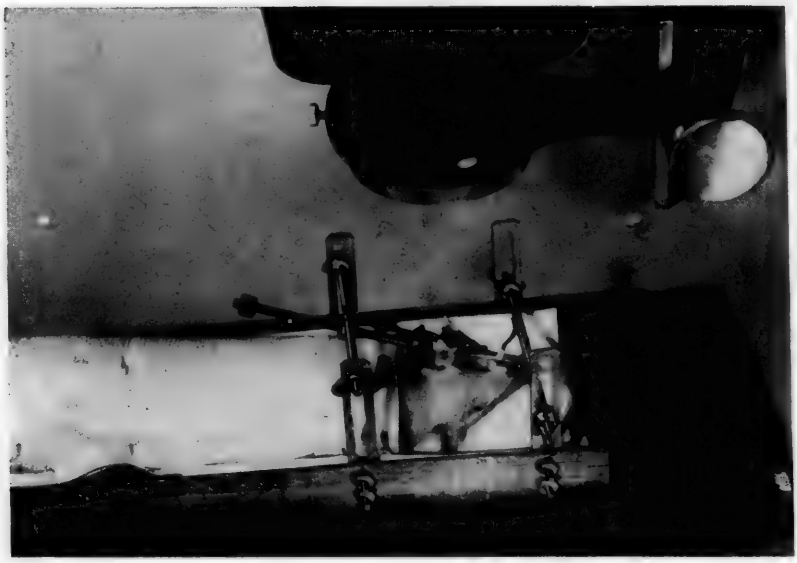

Fig. 3.—Anesthetized rat fixed on the operating table in position for the operation. Note the width of the mouth opening, position of the jaws which are fixed, and the manner of immobilizing the body by means of the towel.

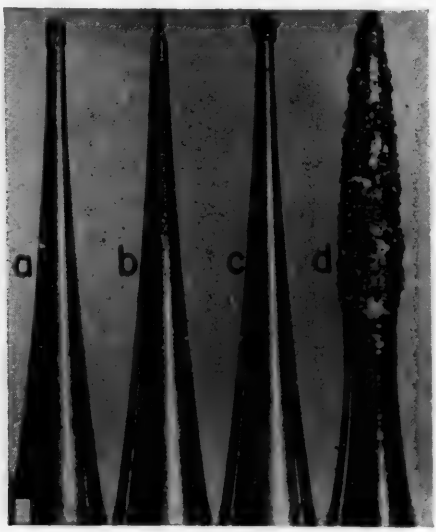

Fig. 4.—Enlarged view of cavity preparation instruments: *a* and *b*, bibeveled drills made by grinding head of No. 33½ inverted cone dental bur; *c*, No. 33½ inverted cone dental bur; *d*, No. 1 Kerr Dental Manufacturing Company dental diamond-point stone.

made part of the operating table, facilitated lateral retraction of the cheek and the focusing of a spotlight upon the operating area. The materials, parts, and dimensions of the operating table are shown in Fig. 2.

Anesthesia.—Sodium Nembutal proved to be the anesthetic of choice. A fresh solution was made on the day it was used. The solution was made according to the following formula:

Sodium Nembutal 0.10 Gm.
Absolute alcohol 1.00 c.c.
Distilled water 9.00 c.c.

One-half centimeter per 150 grams body weight was injected intraperitoneally. Anesthesia was deep and lasted for approximately one hour, which was more than required to complete the operation.

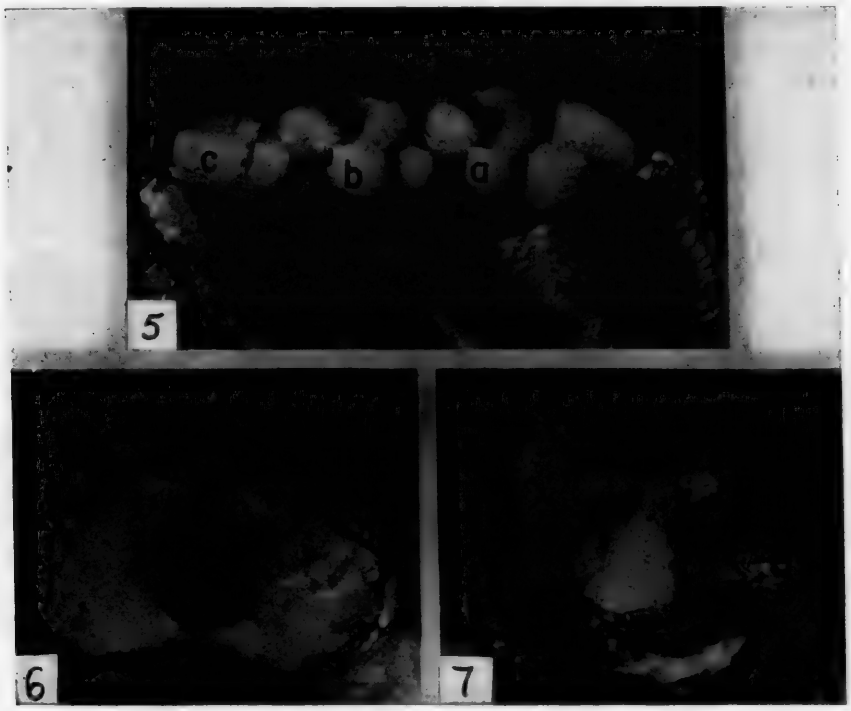

Fig. 5.—Enlarged view of maxillary right molars of the rat: *a*, first molar; *b*, second molar; *c*, third molar. Note the relative size and deep occlusal grooves.

Fig. 6.—Enlarged view of the mesial surface of the maxillary right first molar with the finished cavity preparation. Note the position of the cavity and its size relative to that of the mesial surface.

Fig. 7.—Enlarged view of the mesial surface of the maxillary right first molar with filled cavity.

Preparation of the Animal.—In order to minimize the body movements of the unanesthetized animal, its legs were taped to the thorax and abdomen by means of adhesive tape. The animal was then placed on the operating table

and the mouth opened to the desired width. This was accomplished by placing the incisor teeth of each jaw inside the maxillary and mandibular incisor wire loops of the operating table and adjusting the screw controls (Fig. 3). The right cheek of the animal was then retracted by means of the cheek retractor. A powerful light was focused on the site of operation on the molar, and the eyepiece of the dissecting microscope centered and focused. The use of the dissecting microscope was necessary for adequate magnification of the site of operation.

Cavity Preparation.—The No. 1 Kerr diamond stone was inserted in the handpiece of the dental engine. The tip of the diamond point was placed upon the mesial aspect of the molar midway between the buccal and lingual cusps just above the marginal gingiva. The diamond point was revolved slowly and kept in contact with the tooth until it penetrated the enamel and just reached the underlying dentin. The cavity in the dentin was finished by means of the specially made steel drill, which was rotated by hand with only sufficient finger force to effect cutting. The dentin was easily cut by this means with only minimal frictional heat. When completed, the cavity preparation (Fig. 6) in each instance was approximately 0.5 mm. in diameter and 0.2 to 0.25 mm. deep. At this depth, pulp exposure was not likely, and yet sufficient retention was established for a filling. The cavity was cleaned with water by means of wet cotton wool, dried with air, and filled (Fig. 7). As a trial material a filling consisting of a zinc oxide base was used.[1]

Histologic Preparation.—At the end of each experimental period the animals were sacrificed with ether, decapitated, and the heads were immediately placed in Zenker's formol solution for fixation. Following decalcification and dehydration, the specimens were embedded in celloidin or paraffin. The sections were cut in an anteroposterior direction in a plane that passed from the bifurcation between the anterior and the mesiolingual root through the distobuccal root. For purposes of comparison, a few of the specimens were sectioned transversely. For each specimen the sections were stained with hematoxylin and eosin, Mallory's triple connective-tissue stain, and silver impregnation.

For relatively rapid and preliminary studies, frozen sections can be employed.

SUMMARY AND CONCLUSIONS

A method of cavity preparation in the molar of the rat was developed and standardized. This method proved to be effective for testing the pulpal response of the molar of the rat to different filling materials and medications, and will facilitate further research on the biology and physiology of the pulp as well as on the effects of restorative materials.

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STUDIES ON THE INCORPORATION AND RELEASE OF N^{15} BY TISSUE PROTEINS OF RATS FED N^{15}-GLYCINE

I. The Protein Metabolic Activity of Salivary Glands

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SEVERAL reviews[1-5] in recent years have given the basis for the concept of a dynamic state existing among the tissue proteins of the body. Much, but by no means all, of the evidence has come from the use of isotope-labeled amino acids. For the purpose of introducing the present work it was thought desirable to summarize some of the relevant facts obtained in this manner. It is hoped that such a brief outline will serve an additional function of illustrating why it appeared fruitful to apply this particular approach to the study of protein metabolism of oral tissues.

It has been demonstrated that after the in vivo administration of various labeled amino acids, the relative rate and extent of incorporation into tissue proteins are dependent upon certain properties of the individual tissues. In general, those "tissues which produce protein containing secretions or which have a large store of labile proteins rapidly take up the label."[6] The relative rate of uptake of amino acids by a particular tissue is qualitatively independent of the form in which the label is attached. For example, after administration of S^{35}-methionine,[6] N^{15}-glycine,[7] or C^{14}-tyrosine[8] to experimental animals, it was found that liver proteins always had a far greater activity than muscle proteins. It is not unequivocally known whether amino acid incorporation represents de novo protein synthesis, but it is generally agreed that amino acid uptake is intimately associated with some phase of protein synthesis.[9] Shemin and Rittenberg[7] have defined the uptake of tagged amino acids by tissues "as a measure of the over-all chemical activity or rate of regeneration of the proteins of specific organs or tissues." Generally, amino acid uptake in vitro is inhibited by those agents which block oxidative phosphorylation and by anaerobiosis.[9] However, no apparent correlation has yet been shown to exist between tissue uptake of labeled amino acids and tissue concentrations of various oxidative enzymes. For example, heart muscle has the greatest concentration of cytochrome oxidase,[10] and yet has a relatively low in vivo uptake of N^{15}-glycine.[11]

In summary, while there is no doubt that incorporation of labeled amino acids into tissue proteins is associated with some aspect of cellular metabolism, the mechanism remains obscure.

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Reports which have established these facts have been primarily concerned with the study of protein synthesis, or protein metabolism of such organs as the liver in relation to the entire organism. No reports could be found concerning the relative amino acid uptake by salivary glands, tongue, masseter, oral mucosa, alveolar bone, teeth, and incisor pulps. It was hoped that by obtaining data on these tissues, it would be possible to compare the protein metabolic activity of oral structures with that of the entire organism. The approach used was essentially that of Schoenheimer and Ratner[1, 2] who developed the technique of determining metabolic activity by first administering isotope-labeled compounds to experimental animals and then following the rate of decrease or increase of isotope concentrations in various tissues with time.

EXPERIMENTAL METHODS AND PROCEDURES

Glycine,* containing 31.9 atom per cent excess N^{15}, was used as the amino acid in all experiments. Six cubic centimeters of a 2.5 per cent solution was administered orally in 6 separate feedings over a period of 3 days to male Sprague-Dawley rats weighing between 180 and 220 grams. During this time, and for approximately 2 weeks previously, the animals were maintained on a 15 per cent casein diet† ad libitum. As soon as the N^{15}-glycine feedings were begun, rats were housed in separate metabolic cages, and urinary nitrogen excretion was determined for the remainder of the experimental period. On a few rats fecal nitrogen excretion was also measured.

Until the last N^{15}-glycine feeding was completed, all rats were treated identically. At this time (time zero), the animals were separated into 2 main groups, one of which continued to receive the 15 per cent casein diet, and the other of which was fasted. Rats were sacrificed at varying periods of time, ranging from 10 to 161 hours after the last N^{15}-glycine feeding. All the animals from the fed group were sacrificed at either 18 or 161 hours.

Treatment of Tissues.—At the time of sacrifice the rats were anesthetized with Nembutal (4 mg. per 100 Gm., i.p.) and exsanguinated from the aorta. Selected organs and tissues (liver, chief submaxillary glands, parotid glands, heart, masseters, biceps femoris, etc.) were dissected out, washed free of blood with distilled water, and then immersed in cold trichloroacetic acid (TCA). The nonprotein nitrogen was obtained by twice extracting minced tissues with an excess of cold 10 per cent TCA, washing once with water, and twice with absolute alcohol at room temperature over a period of 1 hour. The alcohol and trichloroacetic acid filtrates were kept separately. Residues were then placed in an oven maintained at 100° C. for a minimum of 48 hours, after which they were weighed and analyzed for percentage nitrogen by the micro-Kjeldahl procedure.

Analysis for N^{15} Concentrations.—Ammonia collected in boric acid by the micro-Kjeldahl procedure was converted to gaseous nitrogen in a vacuum sys-

*The N^{15}-glycine was synthesized by contract by Dr. A. W. Sloan of the Atlantic Research Corporation, Alexandria, Va.

†The percentage constituents of this diet were casein—15; cornstarch—47; lard—23; Wesson oil—4; cod liver oil—2; Brewer's yeast—5; Osborn-Mendel salts No. 1, obtained from Fisher Scientific—4.

tem, after which the nitrogen was introduced into a mass spectrometer for determination of its N^{15} concentration. The methods used were essentially those given by Keston, Rosebury, Rittenberg, and Schoenheimer.[12] Two mass spectrometers were used in the course of this work. One, the Consolidated-Model 103,* was employed during the earlier phases and was found to have a standard deviation of approximately 0.01 atom per cent excess N^{15} in the ranges of N^{15} used. However, readings as high as 0.03 atom per cent excess N^{15} were obtained with known samples containing no excess N^{15}. Subsequently, a Consolidated-Nier, Model 21-201, isotope-dilution mass spectrometer was employed. The standard deviation for this instrument was 0.001 for known samples containing 0.149 atom per cent excess N^{15}. With samples containing no known excess N^{15}, readings did not exceed 0.007 atom per cent excess N^{15}. Within the range of the 2 instruments, duplication was good. However, because of the greater accuracy of the isotope dilution mass spectrometer, the data to be reported here were those obtained primarily with the latter instrument.

Results are expressed in atom per cent excess N^{15}, which is calculated from the ratio determined by mass spectrometry. The method of calculation is that given by Keston, Rosebury, Rittenberg, and Schoenheimer,[12] namely, atom per cent excess $N^{15} = \frac{100r}{2-r} - 0.36$, where r is the ratio of 29 to 28 peaks, and 0.36 is the atom per cent N^{15} of naturally occurring nitrogen. In order to facilitate comparisons, the N^{15} concentrations of various tissue proteins are also expresed as a function of the N^{15} concentration of liver proteins. Liver was chosen as the reference point because its proteins have a high uptake of N^{15}-labeled amino acids, and it has been studied in many other investigations of tagged amino acid incorporation. This relative way of presenting data rapidly shows where the activities of each set of tissue proteins rank in the hierarchy of activities of other proteins from rats maintained under various conditions.

RESULTS

General Balance Studies: Weight.—During the time of acclimatization to the casein diet, and during the N^{15}-glycine feedings, all animals continued to gain weight. Table I summarizes the weight gains during casein feeding, and weight losses during fasting. Since 10 to 14 days elapsed between the introduction of the casein diet and the last N^{15}-glycine feeding, it can be calculated that there was an average weight gain of approximately 20 grams per rat per week, indicating that the casein diet was adequate.

Nitrogen Excretion.—The rats adjusting to a 15 per cent casein diet excreted an average of 125 mg. urinary nitrogen and 8.4 mg. fecal nitrogen per day per animal. Each animal consumed approximately 10 Gm. of food per day, containing 2.33 per cent nitrogen. When the rats were deprived of food, the nitrogen excretion tended to decrease slightly, as seen in Table II. As Tables I and II indicate, there was a 30 per cent decrease in body weight (approximately

*These analyses were performed by contract by the National Bureau of Standards, Division of Mass Spectrometry.

TABLE I

WEIGHT CHARTS OF NORMAL RATS

	GROUP I—FASTED			GROUP II—FED	
Time of sacrifice in hours	18	90	161	18	161
Total number of rats	18	3	12	5	8
Average beginning weight	164	163	160	154	151
Range	(135-189)	(158-168)	(153-168)	(152-156)	(145-165)
Average weight after last N^{15}-glycine feeding	210	202	204	187	202
Range	(185-220)	(195-204)	(190-215)	(182-193)	(184-207)
Average weight at end of experimental period	202	164	142	190	224
Range	(180-214)	(148-172)	(130-161)	(185-195)	(198-255)
Average percentage weight loss since last N^{15}-glycine feeding	-3.8	-18.8	-30.4	+1.6	+10.9

60 grams) after a 161-hour fast, during which each animal lost an average of 650 mg. urinary nitrogen. Fecal nitrogen excretion was negligible during starvation.

TABLE II

NITROGEN BALANCE IN NORMAL RATS FED N^{15}-GLYCINE

	GROUP I—FASTED			GROUP II—FED	
Time in hours between last N^{15} feeding and sacrifice	18	90	161	18	161
Diet after last N^{15} feeding	Fasted	Fasted	Fasted	Fed casein	Fed casein
Total number of rats	18	3	12	5	8
Number of separate groups analyzed	6	1	3	1	1
Average total urinary nitrogen excretion per rat per day, in mg.	125.0 ± 10.1*	109.5 ± 27.9*	110.4 ± 9.7*	135.0 ± 7.8*	122.5 ± 16.3*
Average total urinary N^{15} excretion per rat, from initial N^{15} feeding until sacrifice, in mg.†	1.76 ± 0.24*	2.54	3.15 ± 0.15*	1.96	2.98
Percentage of administered N^{15} excreted in urine	19.4 ± 2.7*	27.9	34.6 ± 1.7*	21.6	32.7

*Standard deviation from the mean.

†The urinary N^{15} excretion was obtained by multiplying the total nitrogen excretion during a given time interval by the N^{15} concentration of the total urine during that period. While the N^{15} concentrations of total urine decreased with fasting time, they were at all times greater than the theoretical "average isotope concentration" as defined by Shemin and Rittenberg.[7] The total urinary N^{15} excretion was then obtained by adding the amounts of N^{15} excreted during different time intervals from the first N^{15}-glycine feeding until sacrifice.

N^{15} Excretion.—Each rat received a total of 150 mg. of 31.9 atom per cent excess N^{15}-glycine, which corresponds to 9.09 mg. N^{15}.* Table II and Fig. 1 summarize what percentage of the administered N^{15} was excreted by rats from

* $\frac{14.32}{75.36} \times 150 =$ total mg. nitrogen

(31.9%) (total mg. N) = mg. excess N^{15} administered.

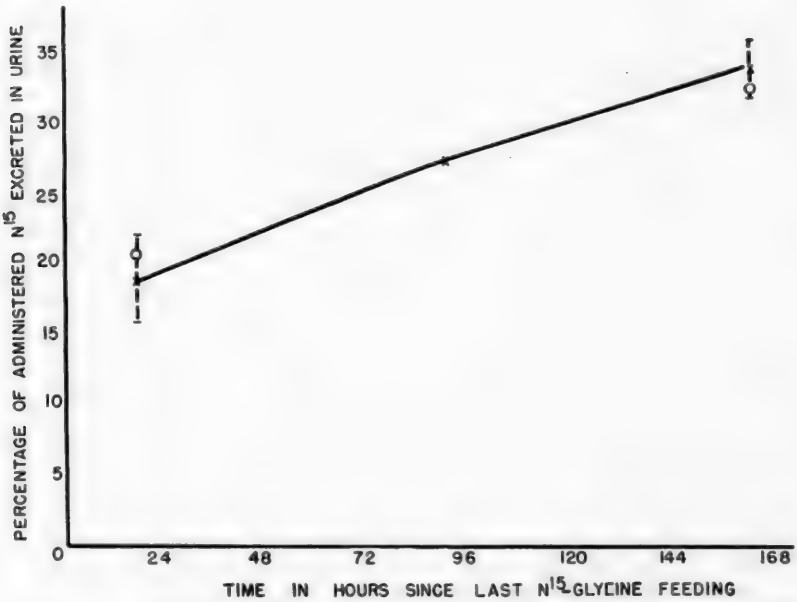

Fig. 1.—*X* symbols represent average total percentage excretion by normal fasted rats; *O* symbols represent the average total percentage excretion by normal fed animals. Bars on either side of *X* symbols indicate standard deviation from the mean. Fasting apparently did not alter percentage excretion of administered N^{15}-glycine.

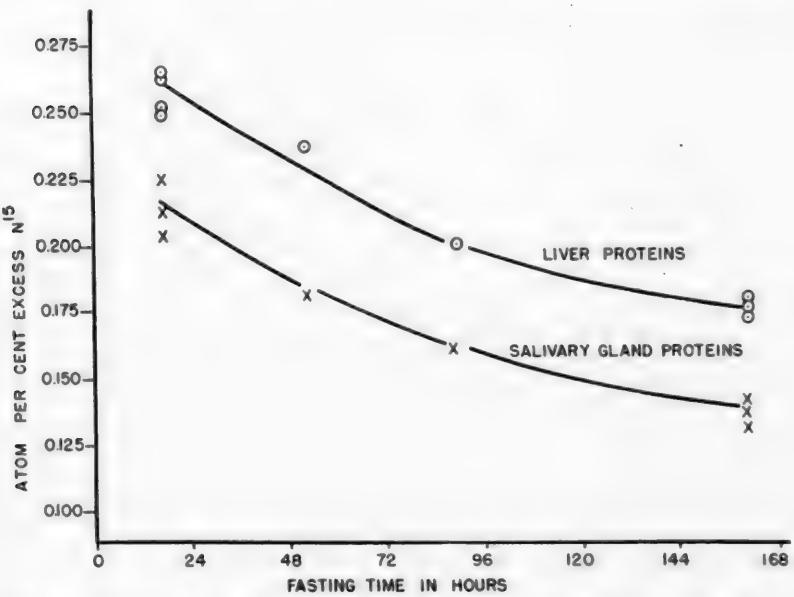

Fig. 2.—Changes in N^{15} concentrations of salivary gland and liver proteins with fasting time, based upon data presented in Table III.

various groups, and also lists total urinary nitrogen excretion by these animals. While no attempt was made to account completely for the N^{15} administered to all rats, experiments were performed on 3 groups of rats to determine precisely where the total incorporated N^{15} was distributed in various organ and tissue proteins from rats fasted 18, 90, and 161 hours, respectively, after the last N^{15} feeding. Details of this complete balance will be presented in a subsequent publication, along with an attempt to account for the sources of nitrogen excreted during fasting. The nitrogen contributed by the salivary glands during fasting is given in detail in the following sections.

Protein Metabolic Activity of Salivary Glands: N^{15} Uptake and Disappearance.—The data in Table III and Fig. 2 demonstrate that the N^{15} concentrations of proteins from pooled parotid and submaxillary glands decreased with time. The lowest concentrations were found in the proteins of those animals which were allowed to continue eating. Undoubtedly, the exogenous nitrogen in the foodstuff diluted the N^{15} which was incorporated into salivary gland proteins, thereby lowering the N^{15} concentrations. However, even when no exogenous foodstuff was permitted, it is evident that the concentration of N^{15} in these tissue proteins decreased with time.

TABLE III
N^{15} CONCENTRATIONS OF SALIVARY GLAND PROTEINS

TIME AFTER LAST N^{15}-GLYCINE FEEDING IN HOURS	DIET OF RATS	NUMBER OF RATS POOLED FOR ANALYSIS	ATOM PER CENT EXCESS N^{15} SALIVARY GLANDS	RATIO OF LIVER A%E N^{15} TO SALIVARY GLANDS A%E N^{15}
18	None	5	0.213	1.23
18	None	5	0.208	1.20
18	None	5	0.229	1.17
52	None	1	0.182	1.30
90	None	3	0.162	1.23
161	None	5	0.138	1.28
161	None	4	0.142	1.30
18	Casein	3	0.220	1.28
161	Casein	4	0.107	1.22
161	Casein	4	0.105	1.21

The ratio of atom per cent excess N^{15} of liver proteins to the atom per cent excess N^{15} of salivary gland proteins is always greater than one. It should be noted that the ratio is maintained within narrow limits over the time intervals studied from 18 to 161 hours. This phenomenon is clearly depicted in Fig. 2, where it can be seen that liver proteins have greater N^{15} concentrations than do salivary gland proteins, and that the relative differences are maintained for the duration of the experiment.

Since Baker[13] had shown histologically that parotid and submaxillary glands respond differently to starvation, it was necessary to compare the metabolic activities of these 2 tissue proteins. Results of experiments designed to see if such differences existed are shown in Table IV. In individual animals tested the N^{15} concentration of submaxillary gland proteins was greater than that of parotid gland proteins, although the differences were small. The rate of decrease of N^{15} concentration with time was not different for the two sets of proteins.

TABLE IV

PROTEIN N^{15} CONCENTRATIONS OF PAROTID AND SUBMAXILLARY GLANDS

FASTING TIME IN HOURS	ANIMAL NUMBER	ATOM PER CENT EXCESS N^{15}	
		PAROTID	SUBMAXILLARY
18	N-1a	0.216	0.226
18	N-1b	0.213	0.223
18	N-1c	0.183	0.213
90	N-2a	0.162	0.168
90	N-2b	0.148	0.156
90	N-2c	0.163	0.171
161	N-3a	0.123	0.130
161	N-3b	0.131	0.142
161	N-3c	0.124	0.134

Fig. 3 depicts the N^{15} concentrations of various tissue proteins from a single rat fed N^{15}-glycine, and shows the relationship of the protein metabolic activity of salivary glands to the activities of other tissue proteins.

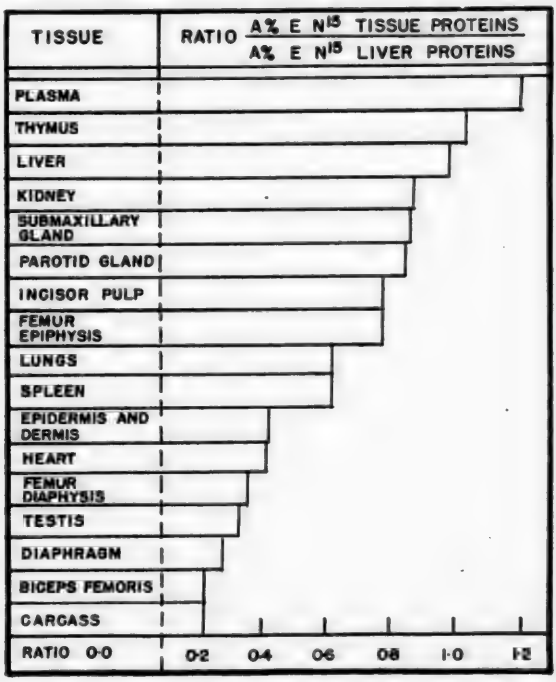

Fig. 3.—Relative chemical activities of tissue proteins from rat fasted 18 hours after last N^{15}-glycine feeding.

Loss of Total Salivary Gland Nitrogen During Fasting.—Results from nine rats are presented in Table V and Fig. 4. It can be seen that the two different glands responded differently to the stimulus of starvation. The parotid glands contributed very little nitrogen during the early stages, but then lost approximately 20 per cent of their initial nitrogen from the ninetieth hour to the one

hundred and sixty-first hour of fasting. In contrast, the submaxillary glands lost over 25 per cent of their initial protein nitrogen during the early stages of fasting and then tended to regain nitrogen with further starvation.

TABLE V

NET PROTEIN METABOLIC ACTIVITY OF SALIVARY GLANDS

ANIMAL NUMBER	FASTING TIME IN HOURS	TOTAL MILLIGRAMS PROTEIN NITROGEN		AVERAGE ATOM PER CENT EXCESS N^{15}		AVERAGE TOTAL MICROGRAMS EXCESS N^{15}	
		PAROTID	SUBMAXILLARY	PAROTID	SUBMAXILLARY	PAROTID	SUBMAXILLARY
N-1a	18	3.62	10.06	0.216	0.220		
N-1b	18	3.63	10.42	0.213	0.223		
N-1c	18	4.46	9.44	0.183	0.213		
Average	18	*3.90*	*9.97*	*0.204*	*0.219*	*7.96*	*21.90*
N-2a	90	3.84	6.87	0.162	0.168		
N-2b	90	3.67	8.11	0.148	0.156		
N-2c	90	3.60	6.59	0.163	0.171		
Average	90	*3.70*	*7.19*	*0.158*	*0.165*	*5.85*	*11.88*
N-3a	161	3.65	8.23	0.123	0.130		
N-3b	161	2.07	7.29	0.131	0.142		
N-3c	161	3.21	9.27	0.124	0.134		
Average	161	*2.98*	*8.26*	*0.126*	*0.135*	*3.78*	*11.21*

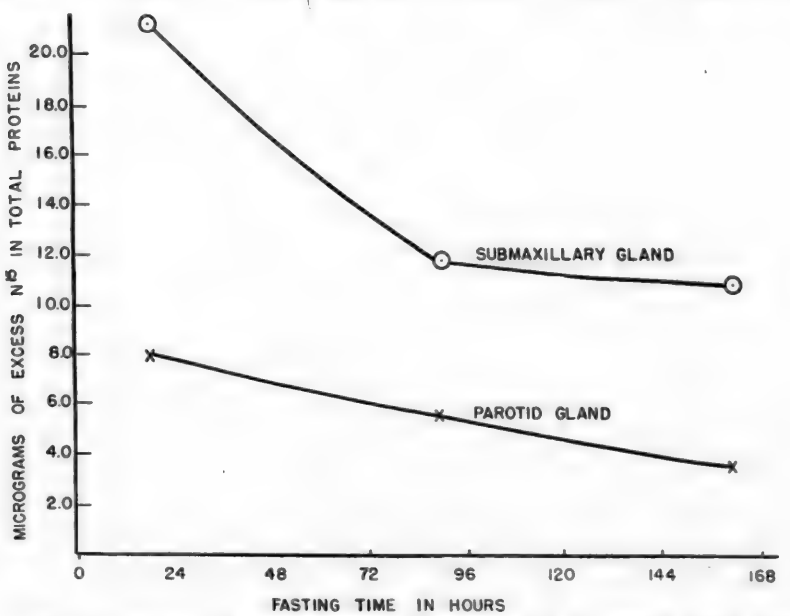

Fig. 4.—Changes of total micrograms of excess N^{15} in salivary gland proteins with fasting time. Each symbol represents the average of data obtained from the tissues of 3 animals.

DISCUSSION

After N^{15}-glycine was administered to Sprague-Dawley rats, a large percentage was incorporated into body proteins, in agreement with the findings of Schoenheimer,[2] Ratner and Schoenheimer,[1] and Shemin and Rittenberg.[7] The

fact that more was incorporated than in previously reported experiments[7] may be attributed in part to differences in the dietary history. The present animals were adjusted to a 15 per cent casein diet, while the rats in previous reports were not. Sprinson and Rittenberg[14] have demonstrated that dietary composition affects uptake of N^{15} compounds, with a low-protein diet increasing the N^{15} uptake, and vice versa.

The decrease in N^{15} concentrations of the proteins of salivary glands with time is a complex phenomenon. If the administered N^{15} were homogeneously distributed throughout the tissue proteins studied, and if no nitrogen from other organs were transferred to the salivary glands during starvation, no change in N^{15} concentrations with time would result, because N^{15} and total nitrogen would be lost in equal proportion. However, the data demonstrate a more rapid decrease of N^{15} than of total nitrogen, thereby invalidating one or both of these assumptions. It is known from the work of Shemin and Rittenberg[7] that the various amino acids of tissue proteins from rats fed N^{15}-glycine have different N^{15} concentrations. It is, therefore, apparent that even in a single protein from a single tissue, the N^{15} may be heterogeneously distributed. The falsity of the other assumption, that organs do not transfer compounds among each other, is demonstrated by various reports indicating the dynamic state existing among the tissue proteins of the body.[1-3]

Explanations for a decrease in N^{15} concentration of tissue proteins must include: (1) the possibility that there is a preferential catabolism of those portions of tissue proteins recently synthesized; (2) the possibility that particular tissue proteins are diluted by proteins from other organs having a lower N^{15} concentration; and (3) the possibility that both these processes are occurring in various degrees.

Under the condtions of these experiments, no tissue proteins studied, except those of incisor dentin,[15] showed an increase in N^{15} concentration with fasting time. Concomitantly, there was no net anabolism of tissue proteins. It, therefore, appears reasonable to hypothesize that recently synthesized portions of proteins were preferentially catabolized during starvation. This interpretation of the data is strengthened by the findings[16, 17] that there is a preferential loss of certain liver enzymes during protein depletion, and that these enzymes are preferentially synthesized when protein is aded to the diet.

Each set of tissue proteins studied had a consistently characteristic uptake and release of isotope. The rate of this "turnover" is supposedly associated with the rate of regeneration of various proteins,[6, 7] but this concept has been recently criticized.[18] The viewpoint has also been expressed that the turnover rate is associated with "lability" of tissue proteins,[6] where "lability" is defined as the percentage nitrogen loss during starvation.[19] However, percentage nitrogen loss is not directly correlated with the percentage decrease in initial N^{15} concentrations, as can be seen from the following data on the nitrogen changes in various tissue proteins. The loss in total salivary gland nitrogen over the time period studied was no more than 25 per cent, while the loss in N^{15} concentration was approximately 35 per cent. Other data[11] indicate that the

thymus proteins decreased by over 70 per cent, while the N^{15} concentrations decreased by less than 45 per cent during 161 hours of fasting. In contrast to thymus proteins, total plasma proteins decrease only slightly during starvation,[19] but the N^{15} concentrations of plasma proteins decreased by approximately 40 per cent. It is, therefore, clear that no necessary direct correlation exists between "turnover rate" and "lability" of tissue proteins, as they have been defined here.

While it is apparent that the N^{15} concentrations of tissue proteins reported here are only average measurements of one aspect of the protein metabolic activity of all the proteins of a given organ, it is nevertheless striking that the relationships among the activities of various tissue proteins are highly reproducible. Fig. 3 shows these typical relationships in one animal fasted for 18 hours. Qualitatively, with minor exceptions, these relationships were maintained throughout the experimental period studied, as can be seen in Fig. 2 in the cases of liver and salivary gland proteins. The tissue protein N^{15} concentrations, then, are an over-all measure of the relative protein metabolic activity of various tissues and organs. The nature of the processes of amino acid incorporation remains obscure. Further elaboration must await a better understanding of the steps of protein synthesis, and knowledge of the metabolic interrelations of individual organs and tissues.

SUMMARY AND CONCLUSIONS

In agreement with previous reports, a large portion of the N^{15} fed as N^{15}-glycine to rats was incorporated into various proteins. Each tissue protein studied had a characteristic uptake and disappearance of isotope. N^{15} concentrations of salivary gland proteins remained quantitatively less than those of liver proteins throughout the experimental period of 7 days. The rate of decrease of N^{15} concentrations of submaxillary gland proteins with time was approximately equal to that of parotid gland proteins, but the initial uptake was consistently slightly higher in the submaxillary than in the parotid glands. Possible interpretations of the data were discussed, and it was tentatively concluded that preferential catabolism of recently synthesized protein, together with possible dilution of salivary gland proteins by proteins from other organs, was associated with the demonstrated decreases in N^{15} concentrations with fasting time.

The author wishes to thank Dr. Julius White of the National Cancer Institute for the use of the isotope dilution mass spectrometer, and to acknowledge the kind cooperation of Mr. William Comstock in performing some of the N^{15} analyses.

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STUDIES ON THE INCORPORATION AND RELEASE OF N^{15} BY TISSUE PROTEINS OF RATS FED N^{15}-GLYCINE

II. The Activities of Muscle, Oral Mucosa, and Incisor Pulp Proteins

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IN A previous report,[1] the protein metabolic activity of salivary glands in relation to the protein metabolism of the whole organism was presented and discussed. The present paper is concerned with the relative protein metabolic activities of other oral, noncalcified tissues obtained from the same normal fasted rats.

EXPERIMENTAL METHODS AND PROCEDURES

The methods of maintaining, housing, feeding, and sacrificing the animals, as well as the methods for removing nonprotein nitrogen and analyzing tissue proteins for nitrogen content and N^{15} concentrations, were identical to those previously reported.[1] The tissue and organ proteins selected for analysis were incisor pulp, palate mucosa, gingival mucosa, tongue membranes, and the following muscles: masseter, tongue, biceps femoris, diaphragm, and skin. Most of these tissues were dissected from the animal at the time of sacrifice, but incisor pulps were removed from the teeth at the time of extraction, which occurred at varying time intervals after sacrifice. Nondissected heads were kept in a deep freeze until time was available to work up the calcified tissues, and to obtain incisor pulps. *Palate mucosal proteins* were obtained from the palatal tissue extending from the mesial of the first molars to the distal of the third molars, scraped clean of underlying muscle and nerve. *Gingival mucosal proteins* were obtained from the lingual, mandibular mucosa in the molar area, and included the gingival papillae. As much of the adhering muscle as possible was removed by dissection. *Tongue membrane proteins* were obtained from surface tissues scraped off whole tongue with the aid of a scalpel moistened with trichloroacetic acid. The middle third of the underlying tongue was used for analysis of *tongue muscle proteins.* Dissection of *masseter, biceps femoris,* and *diaphragm muscles* requires no additional description. *Skin muscle* was prepared by immersing whole skin obtained from the scapular region into 10 per cent trichloroacetic acid for approximately 30 minutes. After this treatment it was possible to remove easily the subcutaneous fat and fascia. The next underlayer was then carefully peeled away from the dermis, and was shown histologically to be muscle (Figs. 1 and 2).

The numbers of rats used and any variations not reported here are included in the appropriate tables.

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Fig. 1.—Photomicrograph of epidermis and dermis of skin from scapular region of rat. Muscle and subcutaneous fascia have been removed as described in the text. (Magnification ×35, reduced ⅔.)

Fig. 2.—Photomicrograph of muscle peeled away from skin placed in trichloroacetic acid for 30 minutes, as described in the text. (Magnification ×90, reduced ⅔.)

These microphotographs were taken by Milton Cheskis of the Medical Illustration Section, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.

RESULTS

Those graphs and tables of the preceding article[1] which represent weight curves and nitrogen excretion include data obtained from the rats of the present experiments, and are applicable to these animals.

TABLE I

N^{15} CONCENTRATIONS OF ORAL TISSUE PROTEINS

FASTING TIME IN HOURS SINCE LAST N^{15}-GLYCINE FEEDING	NUMBER OF RATS POOLED FOR ANALYSIS	ATOM PER CENT EXCESS N^{15}			
		INCISOR PULP	PALATE MUCOSA	GINGIVAL MUCOSA	TONGUE MEMBRANES
10	1	0.263	0.390	—	—
18	3	0.229	0.304	0.219	0.286
90	3	0.200	0.215	0.151	0.220
161	3	0.166	0.140	0.113	0.129

N^{15} Uptake and Release by Proteins of Oral Mucosa and Incisor Pulp.— The data in Tables I and II indicate the relative turnover rates of various oral tissue proteins obtained from animals fasted for varying lengths of time. It should be noted that all proteins listed in these tables had a relatively high initial uptake of N^{15}, and that the rates of release of labeled nitrogen from the proteins of gingival mucosa, palatal mucosa, and tongue membranes were extremely rapid, while the decline of activity in incisor pulp proteins approximately paralleled that of liver proteins. It can be clearly seen from

TABLE II

RATIO OF $\frac{\text{ATOM PER CENT EXCESS } N^{15} \text{ IN LIVER PROTEINS}}{\text{ATOM PER CENT EXCESS } N^{15} \text{ IN TISSUE PROTEINS}}$

TISSUE PROTEIN	FASTING TIME IN HOURS SINCE LAST N^{15}-GLYCINE FEEDING			
	10	18	90	161
Incisor pulp	$\frac{0.311}{0.263} = 1.18$	$\frac{0.256}{0.229} = 1.12$	$\frac{0.226}{0.200} = 1.13$	$\frac{0.182}{0.166} = 1.10$
Gingival mucosa		$\frac{0.256}{0.219} = 1.17$	$\frac{0.226}{0.151} = 1.50$	$\frac{0.182}{0.113} = 1.61$
Palate mucosa	$\frac{0.311}{0.390} = 0.80$	$\frac{0.256}{0.304} = 0.84$	$\frac{0.226}{0.215} = 1.05$	$\frac{0.182}{0.140} = 1.30$
Tongue membranes		$\frac{0.256}{0.286} = 0.90$	$\frac{0.226}{0.220} = 1.03$	$\frac{0.182}{0.129} = 1.41$

Table II, from the steady increases with fasting time of the ratio of activities of liver proteins to the various tissue proteins, that the rate of decrease of N^{15} concentrations of mucosal tissue proteins is greater than that of liver proteins. With the passage of time, the N^{15} concentrations of liver proteins decreased less rapidly than those of mucosal tissue proteins examined, indicating a faster "turnover rate" of the proteins of gingival mucosa, palatal mucosa, and tongue membranes.

N^{15} Uptake and Release by Muscle Proteins.—The data in Table III demonstrate that tongue muscle proteins had the greatest activity of all skeletal muscles studied, but that the N^{15} concentrations of muscle proteins were relatively low. It should be noted that the N^{15} concentrations of all proteins analyzed failed to increase with fasting time.

DISCUSSION

In agreement with findings on the protein metabolic activity of salivary glands,[1] the N^{15} concentrations of all noncalcified oral tissues analyzed decreased with fasting time. While it is realized that none of these tissue protein preparations represents a single pure protein, certain conclusions about the metabolism of these tissue proteins can be drawn from the data. The proteins of tongue membranes, palatal mucosa, and gingival mucosa had a faster "turnover rate" than any tissue protein studied, where "turnover rate" is defined as the relative rate of decline of N^{15} concentrations of tissue proteins from animals previously fed N^{15}-labeled compounds. Schoenheimer, Ratner, and Rittenberg[2] have postulated that the average half-life of liver proteins, calculated from the rate of turnover of doubly labeled L(−)-leucine, is 7 days. If this is the case, then the half-life of each of the above tissue proteins is less than 7 days, while that of salivary glands[1] and incisor pulp proteins is approximately equal to 7 days. This interpretation of the data is only qualitative, and is based upon the results of Shemin and Rittenberg,[3] who demonstrated that by following the rate of decrease of N^{15} concentrations of liver proteins of rats previously fed N^{15}-glycine, a similar figure of 7 days for the half-life of liver proteins could be obtained.

It should be noted that the proteins from tongue membranes and palate mucosa had higher initial uptakes, as well as turnover rates, than any other tissue proteins studied. This probably reflects the extremely rapid rate of regeneration of the proteins of these tissues, and may be considered to be a chemical manifestation of the known rapid healing ability of oral mucosa after surgery.

TABLE III

PROTEIN N^{15} CONCENTRATIONS OF MUSCLES FROM NORMAL FASTED RATS

FASTING TIME IN HOURS SINCE LAST N^{15}-GLYCINE FEEDING	ANIMAL NUMBER	ATOM PER CENT EXCESS N^{15}				
		TONGUE	MASSETER	DIAPHRAGM	BICEPS FEMORIS	SKIN
18	$N\text{-}1_A$	0.101	0.061	0.080	0.065	0.071
18	$N\text{-}1_B$	0.135	0.066	0.076	0.055	0.076
18	$N\text{-}1_C$	0.111	0.067	0.076	0.061	0.065
90	$N\text{-}2_A$	0.119	0.065	0.081	0.054	0.058
90	$N\text{-}2_B$	0.108	—	0.076	0.070	0.072
90	$N\text{-}2_C$	0.108	0.074	0.079	0.058	0.062
161	$N\text{-}3_A$	0.107	0.066	0.057	0.046	0.052
161	$N\text{-}3_B$	0.108	0.066	0.073	0.054	0.054
161	$N\text{-}3_C$	0.098	0.068	0.076	0.048	0.058

The rapid, continuing decrease of N^{15} concentrations of these tissue proteins indicates that the portions of protein recently synthesized are the first to be exchanged with portions in the tissue fluids, or are the first to be lost during conditions of negative nitrogen balance. Since no pure protein was isolated and analyzed, it cannot be stated whether a portion of a protein molecule was preferentially exchanged, or whether a whole species of protein

molecules was preferentially catabolized. Clarification of these points has obvious important implications in extending the knowledge of protein metabolism of the intact organism. While no net synthesis took place in any of the noncalcified oral tissue proteins studied, it has been shown by various authors[4] that amino acids are incorporated into the tissue proteins of starving animals. It is, therefore, probable that at least part of the decrease in N^{15} concentrations of a particular set of tissue proteins can be accounted for by a transfer of nitrogenous compounds from a site having a lower N^{15} activity of tissue proteins, and vice versa. However, if this type of equilibration were occurring to a considerable extent, it would be expected that there would be an increase of N^{15} concentrations of those tissue proteins, like muscle, which initially have a very low N^{15} uptake. Shemin and Rittenberg[3] found such an increase in carcass proteins of rats which had been fed N^{15}-glycine, and which were subsequently kept on a low-protein diet for varying periods of time until sacrifice. In contrast, in the present experiments with fasted rats, there was no increase in N^{15} concentrations of any carcass proteins analyzed. This suggests that during conditions of negative nitrogen balance, there is relatively less incorporation of nitrogenous compounds from tissue fluids into tissue proteins of peripheral organs, and relatively more release of nitrogenous compounds from carcass tissue proteins. From a net standpoint, this is certainly the case, as evidenced by a continuing decrease of total carcass proteins during starvation.[5] Further, the data of White, Hoberman, and Szego[6] indicate that when fasted animals are fed N^{15}-glycine alone, there is greater than normal incorporation into visceral proteins, while relatively less gets into carcass proteins. In addition, since the N^{15} concentrations of total urinary nitrogen of the animals in these experiments remained at all times during a seven-day fast greater than the theoretical "average isotope concentration,"[1] it can be concluded that during conditions of negative nitrogen balance, there is a net over-all preferential loss of recently synthesized portions of metabolically active tissue proteins.

The data in Table III, of the N^{15} uptake and release by various muscle proteins, show that no apparent qualitative correlation existed between natural relative mechanical activity of muscles in vivo and relative protein metabolic activity, as measured by N^{15}-glycine turnover. The diaphragm is probably the most active skeletal muscle per unit weight in the body, and yet its proteins have a relatively low N^{15} uptake. Skin muscle, which probably is one of the least active muscles, has a protein N^{15} uptake approaching that of diaphragm muscle proteins. The factors which permit tongue muscle to have a consistently greater N^{15}-glycine uptake are unknown.

SUMMARY AND CONCLUSIONS

It was demonstrated that the relative turnover rates of the proteins of gingival mucosa, palate mucosa, and tongue membranes were more rapid than the turnover rate of liver proteins. In contrast, the turnover rate of incisor pulp proteins was approximately equal to that of liver.

No qualitative relationship was found between natural mechanical activity of muscle in vivo and protein metabolic activity, as measured by N^{15}-glycine turnover. It was established that the relative initial uptake of N^{15}-glycine was greatest by the proteins of palate mucosa, followed by the proteins of tongue membranes, liver, incisor pulp, gingival mucosa, tongue muscle, diaphragm, skin muscle, masseter, and biceps femoris, in the order named.

There was no increase in N^{15} concentrations with time of the tissue proteins analyzed. This fact, in conjunction with others presented in the discussion, was used to amplify the previously presented hypothesis that during conditions of negative nitrogen balance, there is an over-all preferential catabolism of recently synthesized portions of metabolically active tissue proteins.

The author wishes to thank Dr. Julius White of the National Cancer Institute for the use of the isotope dilution mass spectrometer, and to acknowledge the kind cooperation of William Comstock in performing some of the N^{15} analyses.

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VITAL STAINING OF CALCIFYING BONE AND DENTIN WITH TRYPAN BLUE

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TRYPAN blue has been used frequently as a "vital dye."[1-4] Its administration is known to produce a generalized vital staining of the ground substance of connective tissues with a special affinity for inflammatory lesions. Trypan blue possesses a hydrophobic-hydrophilic molecular configuration associated with active transfer in living cells and also has been shown to combine with proteins.

It was of interest to determine to what extent trypan blue would participate in the formation of those calcified tissues which were of mesenchymal origin and to determine whether or not those tissues were permeable to this dye after formation and calcification had been completed.

EXPERIMENTAL

A 1 per cent aqueous trypan blue solution (Harleco, C. I. #477 for biologic staining) was used throughout both the rat and monkey experiments. Since trypan blue is a colloidal dye, there is a tendency to form aggregates upon standing, which are rapidly taken up by the reticuloendothelial cells; this automatically reduces the concentration of trypan blue in the serum and the amount of dye available for generalized staining of the ground substances. In order to minimize aggregate formation, a fresh solution was made up immediately before each injection, filtered through double-thickness Whatman No. 1 filter paper and sterilized in a boiling water bath for 45 minutes.

Preliminary experiments at different levels and with different frequencies of administration were performed on 36 rats to determine whether or not the contemplated levels of trypan blue were toxic and to observe the pattern of action. The highest dosage selected was 200 mg. per kilogram of body weight. This was well below the lethal level which, in the case of mice, has been reported to be 400 mg. per kilogram of body weight.[5]

Five rats were used to determine the circulating time of trypan blue in the blood. While the rats were anesthetized with Amytal sodium, an appropriate amount of the trypan blue solution was injected into the femoral vein and blood samples were taken from the cut tip of the tail. About 40 to 50 μl of blood was withdrawn at each time interval in a capillary glass tube. After collection of the samples, one end of the tube was sealed preparatory to centrifugation. Thereafter these tubes were broken against a triangular file at the serum-hematocrit separation line, and the serum drained onto a block of paraffin in

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which small concavities had been previously prepared. A 20 μl plasma aliquot was diluted to 5 ml. with distilled water and the optical density determined at 595 mμ in a Coleman Junior Spectrophotometer. The readings were compared with a standard curve which had been prepared with known amounts of trypan blue in 5 ml. of distilled water. The addition of 20 μl serum from uninjected rats to the standard solutions had been found to cause no alteration in the optical density. Care had to be taken to prevent any hemolysis of red blood cells, since, at the low levels of dye concentration, slight traces of hemolysis altered the results by several per cent.

Two rhesus monkeys were given periodic intravenous injections. Small samples of blood were withdrawn before each injection and at different intervals thereafter. Dye concentrations in the blood serum were determined as in the rat and, in addition, electrophoretic studies were performed on the serum. The first monkey studied was a mature female weighing 6.6 kilograms with all 32 permanent teeth fully erupted. She was injected on the first, sixth, and eighteenth experimental days with 30 mg., 50 mg., and 100 mg. of trypan blue per kilogram of body weight, respectively. This monkey was sacrificed on the twentieth experimental day. The second monkey was an immature male weighing 3.7 kilograms at the beginning of the experiment with the following mixed dentition:

6	e	d	e		1		1	2	b	d	e	6
6	e	d	e	2	1		1	2	c	d	e	6

He was injected on the first, third, sixteenth, and seventeenth experimental days with 50 mg. of trypan blue per kilogram of body weight, and on the fifty-ninth and 142nd experimental days with 100 mg. of trypan blue per kilogram of body weight. The monkey's weight on the 143rd day at the termination of the experiment was 3.9 kilograms.

Blood samples were taken at appropriate times in order to determine the amount of trypan blue in the serum. In the monkeys, individual teeth were extracted at different intervals after the injections for the preparation of histologic sections. The following 3 methods were used for the fixation and sectioning of the teeth: (1) Formalin or "Susa" fixation was followed by trichloroacetic acid decalcification and paraffin embedding. Unstained and eosin counterstained serial sections were prepared. (2) Dioxan or formalin fixation was followed by embedding in methyl methacrylate. The embedded blocks were cut into slabs with a rotating steel saw; these slabs were ground on grinding stones to minimal thickness in preparation for mounting. (3) Formalin fixation was followed by dehydration and cutting the unembedded teeth into slabs with a rotating steel saw. Then thin sections were prepared by free-hand grinding on rotating stones with alcohol lubrication to prevent heating.

Paper electrophoretic studies were performed on the dye solutions and on the blood serum according to Köiw's modification, using Whatman No. 1 filter paper, 0.05 M veronal buffer at pH 8.6. Electrophoretic separation was performed at room temperature on 0.02 ml. blood serum, using 270 V. potential for

4 hours. Azocarmine was used as a protein stain. The electrophoretic curve was obtained by directly reading the stained patterns through a photometric scanning device.

RESULTS

Simultaneously with the trypan blue injection, the monkeys and rats became uniformly blue stained. Within about 15 minutes dye localization was seen in places of any minor injuries or epidermis scaling. Also, in the monkey, the areas became deeply stained which had been lightly bruised during catching or during the holding process.

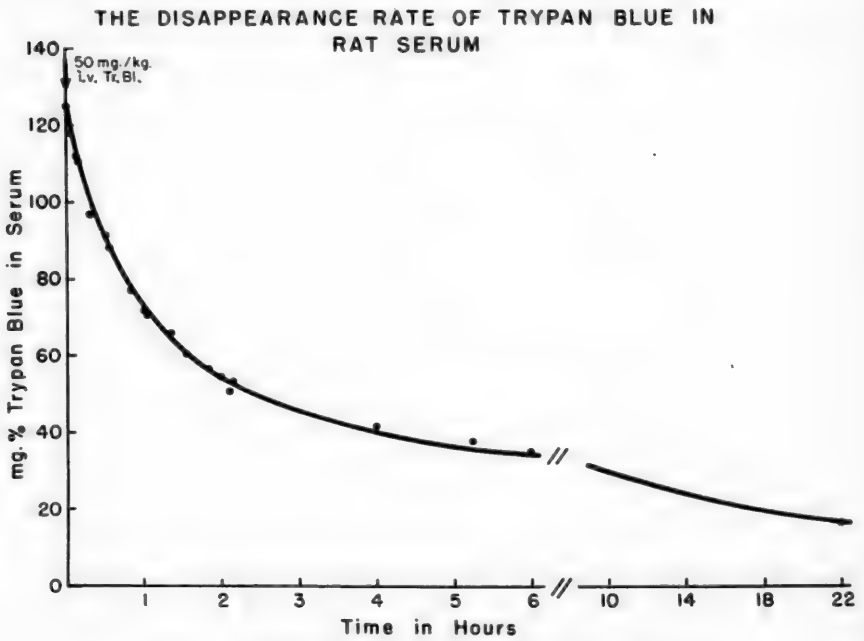

Fig. 1.—Disappearance rate of trypan blue in rat serum.

Injections of 100 mg. of trypan blue per kilogram of body weight were given to 12 weanling Wistar strain rats (average weight 90 grams). These rats gained weight comparable to uninjected controls throughout the period of observation. Rats which had received 200 mg. per kilogram of body weight in two injections 1 week apart grew normally, went through normal reproductive cycles, and delivered healthy litters. Three pairs of rats were mated successfully in which both the male and female rats had had the dye injections. One pair had two healthy litters.

During the first 2 hours, the injected trypan blue very rapidly became reduced in concentration in the serum of the rats and monkeys; thereafter it was cleared at a relatively low rate. Trypan blue was still readily detectable in the serum for as long as 2 weeks after each injection. A curve of the disappearance rate for rats is shown in Fig. 1. The disappearance rate of trypan blue from

the serum was a relatively constant one in both experimental monkeys and rats and the 48-hour values were in the neighborhood of 20 mg. per cent in both species regardless of whether 30, 50, or 100 mg. of dye per kilogram of body weight was injected. These over-all clearance curves represented a fairly slow disappearance of the dye from the circulatory system. This relatively prolonged retention in the serum seems to have enabled a good dye deposition in the tissues.

When the 1 per cent trypan blue solution which was used for injections was studied by means of paper electrophoresis using 0.05 M veronal buffer at pH 8.6, it was separated into 2 fractions, one stationary blue fraction and a purple one, with a slight migration toward the positive electrode. When monkey blood serum was separated electrophoretically before and after dye injection, an interesting phenomenon was observed. After injection, the purple fraction migrated simultaneously with the albumin but at the same time decreased the electrophoretic mobility of the albumin by about 9 per cent as compared with the control serum taken from the same monkey before dye injection. The mobility of the purple fraction in serum was increased more than 10 times the rate observed for the purple fraction of the dye solution alone. This decreased mobility of the albumin fraction and increased mobility of the purple fraction of the dye was observed as early as 45 minutes after the dye injection. The albumin fraction gradually returned to its normal mobility, reaching the control value after 24 hours. These observations suggest that there has been a binding of the albumin fraction of the serum and the purple fraction of the dye. On the contrary the blue portion of the dye was still observed to be stationary and coincided with the position of the globulin fraction. Thus there was no evidence that the blue fraction of the dye was altered by introduction into the blood stream.

Histologic examination of representative tissues of these rats and monkeys showed the anticipated staining of the ground substance of mesenchymal tissues. There was a sharp contrast between the intensely staining subgingival connective tissue and the unstained gingival epithelium, which is of ectodermal origin. A similar contrast was noted between the deeply staining odontoblasts and connective tissue elements of the pulp and the unstained ameloblasts. Likewise the predentin which was forming during the period that the trypan blue was in the serum stained intensely blue (Figs. 4 and 5), whereas the newly forming enamel was not stained. The amount of trypan blue deposited in the predentin closely reflected the concentration of trypan blue in the serum. The predentin formed in the first 24 hours after the trypan blue injection was extremely dark blue, while that formed in the following days became increasingly less blue until the unstained preinjection state was reached. The deposition of bone salts in the predentin zone did not alter the blue staining, but this was maintained as a distinct line in the dentin after calcification was completed. The first lines still reflected the gradation from deep to pale blue attributable to the diminishing dye concentration of the serum (Figs. 2, 4, and 5).

The trypan blue lines in the dentin were separated by distances proportionate to the length of time between the injections (2, 6, and 12 weeks, respectively) in the immature monkey. These lines were noted in the dentin of the incisors of

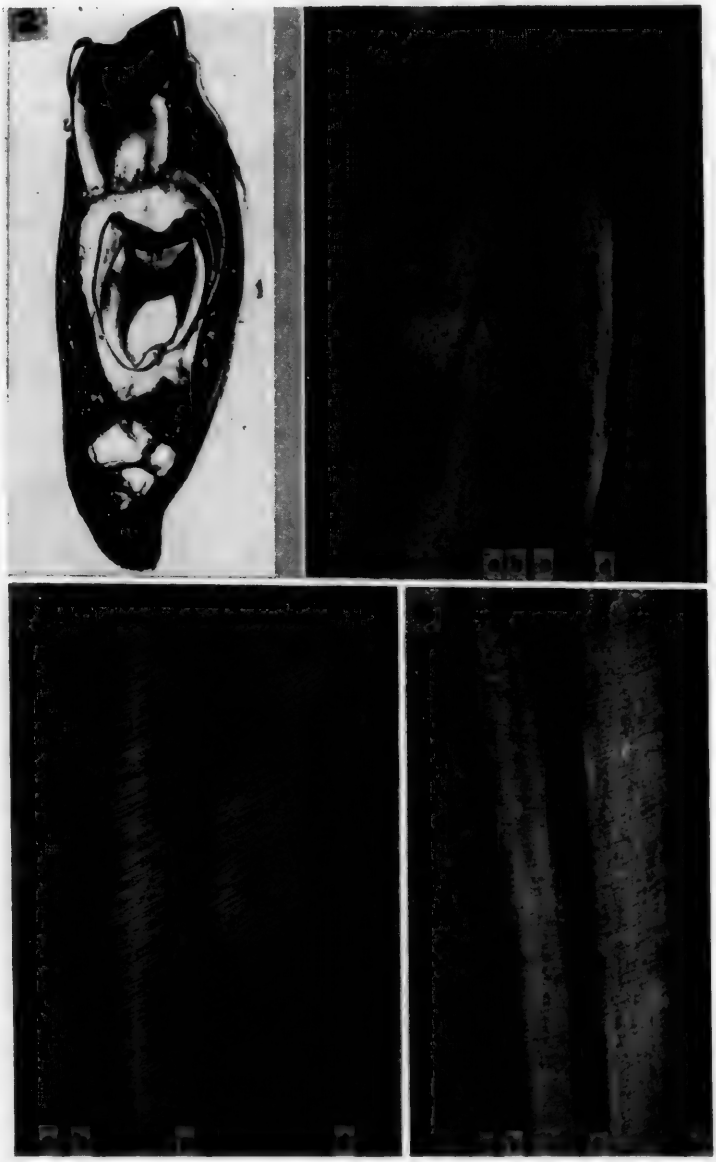

Fig. 2.—Formalin-fixed, undecalcified, ground cross section of monkey mandible in the region of the deciduous molars. (×5; reduced ¼.) Trypan blue is deposited as appositional lines in the cortical bone of the mandible and in the dentin of the unerupted permanent premolar. Osteocytes, bone marrow, periodontal membrane, and subgingival connective tissues have the dye deposit.

Fig. 3.—Portion in the cortical region of specimen in Fig. 2. (×150; reduced ¼.) Dye deposit can be seen as accentuated lines of cortical bone. *a*, *b*, *c*, and *d* represent areas of bone formation following injections on the first, sixteenth, fifty-ninth, and 142nd experimental days.

Fig. 4.—Ground section of an unerupted monkey incisor. (×150; reduced ¼.) Dark lines represent dye deposit in dentin that was forming at time of injection. Line *a* represents area of predentin formation during period after injection of trypan blue on the first experimental day. Lines *b*, *c*, and *d* represent predentin formation after the injections on the sixteenth, fifty-ninth, and 142nd days, respectively. Note the intense staining of the predentin which represents the dye deposited from the injection 1 day prior to sacrificing animal.

Fig. 5.—Section through root portion of forming permanent maxillary incisor. (×150; reduced ¼.) Same intervals between the lines existed as in Fig. 3. Growth pattern of root dentin is well demonstrated.

the rats, and in the root dentin of the rat molars. Comparable lines were observed in the immature monkey in the crown dentin of developing permanent teeth and the secondary dentin of the deciduous molars, and in the root dentin of the permanent teeth of the mature monkey. Wherever there was exposed dentin on the occlusal surfaces of the monkey teeth, there were appreciable accumulations of trypan blue. Although the teeth of both experimental monkeys gave the impression of being blue clinically soon after the injections, no definite visual evidence of a widespread penetration of the dentin or enamel which was fully formed was found, but there was a slight suggestion of a very low concentration of trypan blue in the dentin.

The mandibles of both monkeys were still growing. In the case of the immature monkey, growth was taking place at a relatively rapid rate. Concentric rings of trypan blue were observed in the cortical bone as shown in Figs. 2 and 3. It is noteworthy that the trypan blue lines are much closer together and much less distinct in the cortical bone than in the dentin. The lines due to the first and second injections which were separated by only 2 weeks almost merge. However, comparable spacial relationships between the several trypan blue lines to each other were noted in the mandibular cortex as had been seen in the dentin.

The best observations of the trypan blue lines in the calcified structures were possible with the formalin-fixed, unembedded ground sections. Although these sections were not thin enough for very detailed microscopic studies, the best preservation of the trypan blue lines was noted. In the decalcified sections (7μ and 10μ), the trypan blue lines in the dentin were extremely faint even though the pulp and periodontal tissues contained high concentrations. In the plastic-embedded, undecalcified section the blue in all tissues was converted in the course of the embedding procedure into a very pale purple, which was not nearly as readily visible microscopically.

DISCUSSION

Trypan blue appears to be a good vital stain to be used to measure the rate of growth in developing dentin and bones or to act as a chronologic reference point to mark the stage of growth at which experiments in young growing animals were begun. In the dosages used in these experiments, the health and well-being of the animals seemed to be unaffected, and also the teeth and bone structures formed while trypan blue was in the circulatory system were normal except for the dye deposit in the layers of dentin and teeth that were in the forming stage at the time of injection.

Alizarin red S has been used for an appreciable period as an indicator for the measurement of appositional growth. In the case of alizarin, the dye is bound to the bone salts in such a way that decalcification of the specimens results in a loss of the reference point. In contrast, trypan blue is not lost by decalification, which would suggest that it has a relationship to the organic moiety of the dentin and bone instead of to the bone salts. Since no toxic reactions to the injection of trypan blue were noted, it appears to be preferable to alizarin in this respect.

SUMMARY

Freshly prepared aqueous trypan blue solution was injected intravenously into 36 white rats and 2 rhesus monkeys.

Studies on the rate at which the trypan blue was cleared from the blood indicated that appreciable amounts of this dye were still in circulation for as long as 2 weeks after the injection. Electrophoretic studies demonstrated that the trypan blue was composed of a purple fraction which migrated slowly toward the positive electrode and a stationary blue component. After injection, the purple fraction migrated with the albumin component of the serum and depressed the rate of migration of the albumin. In contrast, the blue portion of the trypan blue remained stationary.

In addition to the generalized staining of the ground substance of the mesenchymal tissues, appreciable amounts of trypan blue were deposited in the areas of the bones and dentin which were being formed while the trypan blue was in the circulatory system. In the dentin in particular, this deposition of trypan blue resulted in strong blue lines which were readily visible in ground sections.

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HASTENING INFILTRATION OF SPECIMENS WITH CELLOIDIN BY BOILING

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THE ordinary method of embedding in celloidin has the disadvantage of requiring a comparatively long time. For large specimens this process may take several months. The rate of penetration is dependent upon the size of the molecules, the viscosity, and the distance. The molecules of the nitrocelluloses are very large and, when dissolved in ether alcohol, they form a very viscid solution. As a result, the rate of penetration is not a linear function of the distance, as is usually the case in solutions. On boiling, however, the rate of penetration is noticeably increased. The celloidin-ether-alcohol solution boils at 44° to 50° C., a temperature which can hardly damage the specimen, since embedding in paraffin takes place at about 56° C.

The celloidin solution cannot, however, boil in an open vessel, because the ether alcohol would evaporate too rapidly. A reflux condensor in which the ether alcohol vapors condense and run back into the vessel is used. The equipment here described has been found to be the most convenient. This setup (Fig. 1), which has been designed for a large laboratory, has 7 condensors mounted in series. The connection between the specimen bottle and the condensor must be absolutely tight. According to my experience this can be attained only by means of a ground joint, preferably one of the standard types B 34 (Figs. 2 and 3). During the boiling, the 2 parts of the ground joint are kept in close contact by means of tight elastic bands fastened on hooks at the outer and inner part. The specimens should not be allowed to lie at the bottom of the vessel, as they may then get stuck, but should be suspended in small steel nips (Fig. 3) which can be attached either to small hooks in the inner part of the ground joint or to threads running through the whole condensor to its upper end. It is an advantage, although not absolutely necessary, to have a thermometer in the bottle (Fig. 2), by means of which the boiling point can always be controlled. Even with a good ground joint some of the ether alcohol will evaporate by long boiling, and this will raise the boiling point. If the temperature rises above 50° C., the bottle should be refilled with ether until the boiling point drops to its original temperature. It is important to keep a 50:50 ratio between ether and alcohol, as too high a concentration of alcohol will give an inferior quality of embedding. If no thermometer is used, a pencil mark may be made on the bottle indicating the fluid level. When the fluid evaporates below this mark, new fluid should be added.

The condensor should be closed with a cotton pellet in order that no moisture may penetrate into the tube to mix with, and spoil, the celloidin solution.

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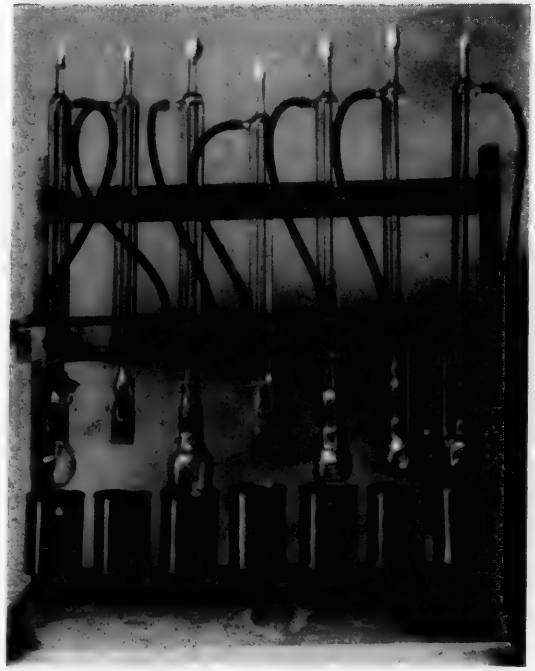

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 1.—Setup, showing bottles combined with reflux condensors by means of ground joints. Condensors mounted in series. Heat generated by 15-watt electric lamps hidden in screens.

Fig. 2.—Detail picture of bottle with ground joint. Thermometer inserted into glass tube connected with bottle. Thermometer fastened with cement on top of tube. Additional tightening by rubber covering.

Fig. 3.—Detail picture showing bottle without thermometer. Specimens suspended in stainless steel nips fastened to hooks in inner part of ground joint. Number tags fastened to nips

The condensor should be at least 50 cm. high. Since the temperature of the coolant should not surpass 15° to 20° C., ice cooling may be necessary in a very warm room. It is advisable to have the apparatus made by an experienced glassblower.

Heat is produced by a 15-watt electric lamp. There is no danger that the ether alcohol will catch fire even if it comes in direct contact with a lamp of this power. The usual precautions associated with the handling of ether must, of course, be taken. A natural objection to this method is that the boiling might facilitate the formation of peroxide, which is highly explosive. Since the presence of peroxide is difficult to determine with certainty in the ether-alcohol-celloidin solution, experiments were made with pure ether, which was boiled under the same conditions. Pure ether gave no trace of peroxide after having been boiled for 5 days. The prevention of peroxide formation must be considered by everyone who works with ether, and on boiling, fresh peroxide-free ether should be used. With tight ground joints and a good condensor, no ether vapor will escape into the laboratory and the boiling may be continued day and night.

For teeth and large pieces of tissue, it is sufficient to boil the specimen for 48 hours in a 4 per cent solution, and for 48 to 72 hours in an 8 per cent solution. Toward the end, the solution should be as thick as possible. The last phase of the embedding process, the hardening, cannot be influenced by the boiling, but must take place in the ordinary way. The specimen is removed from the boiler and is placed in a bottle of proper size. Then, either the solution in which it was boiled or a new thick celloidin solution is poured over it. It is recommended that ample time be used for the final hardening of the celloidin.

Although only the first part of the embedding process may be shortened by this technique, for large specimens this may mean a saving of weeks or even months. I have used the method for 5 years and have found no drawbacks with it. It does not influence the quality of the staining, and the specimens are easy to cut.

SUMMARY

A method is described by means of which embedding in celloidin is performed by boiling the specimen in the celloidin solution. To prevent disappearance of the ether and alcohol, the boiling is undertaken in a bottle which is combined with a reflux condensor by means of a tight ground joint. The celloidin solution boils at a temperature of 44° to 50° C. The heat is produced by means of a 15-watt electric lamp. The usual precautions advised when handling ether should be taken. Considerable time can be saved when large specimens are concerned. No contraindications to this method have arisen during frequent use over a 5-year period.

A SIMPLIFIED METHOD FOR THE DETERMINATION OF CITRIC ACID IN DENTIN AND BONE

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THE conversion of citric acid to pentabromacetone by oxidation and bromination is the basis of a number of methods for its determination.[1-4] The methods all require 2 steps, the formation of the pentabromacetone and the production of a colored derivative that can be estimated. The first step in all the methods requires the use of potassium permanganate, but for the subsequent color development a wide variety of reagents are used—sodium sulfide,[1] sodium iodide,[2] potassium iodide,[3] and thiourea.[4] Recently Ettinger, Goldbaum, and Smith[5] employed the reaction of Fujiwara[6] between trihalogenated hydrocarbons and alkaline pyridine for color development. The use of permanganate in the first step has some disadvantages, particularly the need to maintain a low temperature during the reaction and the length of time required for the subsequent removal of the excess permanganate. To overcome these objections Weil-Malherbe and Bone[7] and Taylor[8] used vanadic acid as an oxidizing agent that could be subsequently rapidly removed at room temperature by the use of ferrous sulfate. We have combined the advantages of the use of vanadic acid as an oxidizing agent with alkaline pyridine as a chromogenic agent to give a method for the determination of citric acid that is rapid, simple, and precise. The method has been applied for the determination of citric acid in dentin and bone, and it can also be applied to blood and urine.

EXPERIMENTAL

Reagents.—

(1) 27 N sulfuric acid. Dilute 750 ml. of conc. sulfuric acid to 1 L. with distilled water.

(2) Bromide-bromate, vanadate (BBV) reagent. One liter of solution contains 19.84 Gm. KBr, 4.92 Gm. $NaBrO_3$, and 12.00 Gm. NH_4VO_3. It may be necessary to warm the liquid to effect complete solution.

(3) 17.6 per cent ferrous sulfate solution. Shortly before use dissolve 8.8 Gm. of $FeSO_4 \cdot 7H_2O$ in about 30 ml. of distilled water to which is added 2 ml. of the 27 N sulfuric acid and then make up to 50 ml. with distilled water.

(4) Potassium hydroxide solution (40 Gm. KOH dissolved in distilled water to make 100 ml. of solution).

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(5) N-heptane, b.p. 98-98.5° C.
(6) Pyridine, analytic grade.
(7) Anhydrous sodium sulfate, analytic grade.

Preparation of Samples for Analysis.—The bone samples were ground to 60 mesh in a Wiley mill. Human coronal dentin was obtained from normal first molars by grinding with a cooled dental bur. The samples were dried to constant weight in a desiccator. Samples of the bone and dentin powders, not exceeding 50 mg., were weighed on an analytic balance and transferred to 100 ml. volumetric flasks. After the addition of 10 ml. of 1 N HCl the flasks were heated to boiling in a water bath. After solution was effected the flasks were cooled and the volume was adjusted to 100 ml. with distilled water. Except for particles of bone and dentin protein the powders dissolved readily. Such solutions contain from 300 to 700 γ of citric acid and a 4 ml. aliquot can be used for analysis.

Standard 1 to 10 tungstic acid filtrates of blood may be used and urine need only be diluted with water to the proper range, not exceeding 10 γ of citric acid per milliliter. Since all trihalogenated hydrocarbons will give the color reaction with alkaline pyridine, trichloroacetic acid cannot be used to dissolve the tissue, nor to precipitate the protein. Likewise, chloroform cannot be used as a preservative.

Method.—To 4 ml. of the sample in a 25 ml. glass-stoppered cylinder is added 4 ml. of 27 N sulfuric acid. The solution is well mixed and allowed to cool to room temperature, and then 4 ml. of the BBV reagent is added and mixed well. The reaction is allowed to proceed at room temperature for 20 to 30 minutes. During this period the ferrous sulfate solution can be prepared. At the end of the oxidation period 2 ml. of the ferrous sulfate solution are added and, as the cylinder is restoppered, a drop or two of the same solution is allowed to flow around the stopper. The cylinder is then shaken briefly and allowed to stand for 10 minutes. Any bromine vapor remaining in the air space above the liquid is removed by a glass tube connected to a water aspirator. There should be no detectable odor of bromine remaining. Six milliliters of n-heptane is then added and the cylinder is stoppered and shaken for one minute. When the layers have separated, the lower aqueous layer is removed with a syringe pipette.* A small amount (not exceeding 0.1 Gm.) of anhydrous sodium sulfate is added to the cylinder through a powder funnel. The cylinder is again stoppered and shaken briefly and the heptane extract is poured into a clean, dry test tube. A 5 ml. aliquot is placed in a 200 by 25 mm. test tube containing 6 ml. of pyridine and 3 ml. of 40 per cent potassium hydroxide and stoppered with a cork covered with lead foil. The test tube is well shaken without allowing the contents to contact the stopper. The test tube is then placed in a water bath at 80° C. for 4 minutes, after which it is quickly chilled in an ice-water bath. The mixture is poured into a colorimeter tube and, when the layers have separated, the lower potassium hydroxide layer is removed with a clean, dry

*The syringe pipette consists of a 25 ml. hypodermic syringe attached by means of a 1-hole rubber stopper to a piece of glass tubing drawn out to a point and long enough to reach to the bottom of the reaction cylinder.

syringe pipette. The colorimeter tube is centrifuged at 2,000 r.p.m. for 10 minutes and the pink color is read in a Klett-Summerson photoelectric colorimeter using a number 54 filter. A reagent blank is similarly prepared for obtaining the zero reading.

Discussion of the Method.—The temperature of the solution during the oxidation with the BBV reagent is important. After mixing with the sulfuric acid, the sample had a temperature of about 55° C. If the BBV reagent is added without allowing the temperature to come to that of the room, a 25 per cent decrease in the yield of pentabromacetone results. It was found desirable to add the BBV reagent, the n-heptane, and also the pyridine from burettes.

In the initial experiments with the method it was found that a cloudiness often developed in the pyridine layer which could not be removed by centrifugation. This cloudiness was found to be due to the presence of water carried over from the reaction mixture. The cloudiness is eliminated by the use of 40 per cent potassium hydroxide instead of the 30 per cent used in the original method of Ettinger, Goldbaum, and Smith.[5] The more concentrated alkali removes the last traces of water.

The color development with the alkaline pyridine appears to be influenced by some constituent of the pyridine. The intensity of the color produced varies with each particular lot of pyridine, and even when the pyridine is double distilled this variation cannot be eliminated. It is necessary, therefore, to run citric acid standards with every determination; the 2 standards that are routinely employed contain 5 γ per milliliter, and 10 γ per milliliter, respectively.

Precision of the Method.—Using a solution of citric acid containing 5 γ per milliliter, 10 determinations were carried out using the permanganate method of Ettinger, Goldbaum, and Smith,[5] and also 10 determinations by the oxidation method described previously. The extraction of the pentabromacetone and the subsequent color development were the same for the 2 sets. The standard deviations were calculated and were found to be $\pm 0.55\ \gamma$ for the method here described and $\pm 0.33\ \gamma$ for the permanganate oxidation, a highly satisfactory degree of precision.

TABLE I

CITRIC ACID CONTENT OF DENTIN AND BONE

MATERIAL	CITRIC ACID		
	1 (PER CENT)	2 (PER CENT)	LITERATURE VALUES (PER CENT)
Dentin	0.86	0.87	0.80 - 0.89[9, 10]
Dog tibia	1.36	1.39	1.31[11]
Monkey tibia	0.76	0.76	
Rabbit humerus	0.64	0.61	

Results.—The citric acid content of dentin and of the samples of bone from several animal species is given in Table I. The values in Column 1 are the averages of duplicate determinations done in one series of analyses, and those in the second column the average of a second series of experiments. It is apparent that the replicates are in good agreement and also agree well with the literature values given in the third column.

SUMMARY

A simplified photometric micromethod for the determination of citric acid has been presented. It is based upon the oxidation and bromination of citric acid to pentabromacetone by vanadic acid in the presence of bromine. The pentabromacetone is then reacted with alkaline pyridine to give a pink-colored pyridine solution. The method has been used for the analysis of bones and dentin and can also be applied to blood serum and urine.

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